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13. ABSTRACT *(Maximum 200 words)*

The Center for Devices and Radiological Health of the Food and Drug Administration has performed research over the past two years with financial support from the Walter Reed Army Institute of Research. This research has addressed three tasks which are described in the report.

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The Center for Devices and Radiological Health of the Food and Drug Administration has performed research over the past two years with financial support from the Walter Reed Army Institute of Research. This research has addressed three tasks which are described below. The details of these studies may be found in the 14 publications listed below. Copes of these publications or abstracts are attached.

This work has addressed the health issues concerning exposure of humans to electromagnetic radiation. These concerns stem, in part, from reported epidemiological studies which have shown an association between exposure to extremely low frequency (ELF) fields and cancer; thus, interest in ELF as a potential tumor promotor has occurred in the scientific community. Cancer cells often express mutated versions of normal cell cycle regulatory genes called proto-oncogenies. An agent such as ELF could cause altered expression of a proto-oncogene and/or other genes which could lead to inappropriate cell proliferation and increase the likelihood of transformation. These concerns are further raised by ELF modulated radiofrequency and microwave radiation. There are reports in the literature to suggest that levels of ODC and calcium may be altered by ELF modulated RF fields. Again, the health consequences are unknown. However, such changes in second messengers could result in effects on gene expression which, in turn, could have adverse consequences.

A summary of results of the 14 attached abstracts or reports is given below for convenience. The reader is referred to the individual reports for details.

Task 1. The objective of the first task was to identify conditions of exposure which produce kinetic effects. Several different cell lines were used, including HL 60, Daudi, human peripheral blood lymphocytes, and GM1500B. These results taken as a whole do not provide a comprehensive picture of dose response. First, we find that there is an apparent cell line dependence to response of c-myc expression when exposed to 60 Hz magnetic fields (Ref. 4, 6, 13, 14). For some dose response measurements there appears to be a threshold of about 0.75 mG (Ref. 6, 8, 14). The variance with other reports in the literature may indicate that more than one mechanism is operative. These issues can only be resolved with more investigation.

Studies of pulsed microwaves showed a pulsed dependence (Ref. 5). Exposure of normal human lymphocytes to continuous wave 2450 microwave fields showed no difference in exposure and controls at non-heating levels. For pulsed exposure there was a non-thermal response. The question remains as to whether this response was due to the peak value of the pulse or due to the ELF modulation

frequency (100 Hz repetition rate). Further studies to resolve this issue will be conducted in the future but are unresolvable at this time due to equipment failures.

Task 2. Effort under this task addressed the mechanistic issues of whether a particular responder gene(s) may be responsible for observed ELF effects on gene expression. Tranfection of Schwann cells with a plasmid containing the SV-40 T antigen under the control of the metal regulatory element (Ref. 8, 10). These results indicate that specific promoter genes may be responsive to ELF fields. Studies indicate that alteration in the promoter used cause it to become non-responsive to ELF fields. Some promotor genes are responsive to ELF fields and other are not. Follow up of these studies may be most important in determining mechanisms of ELF or ELF modulated RF responses.

References 2, 9 and 12 further address mechanisms. It has been suggested that observed effects of ELF fields on cellular systems are membrane mediated processes. It has been further suggested that shifts in the levels of calcium, acting as a second messenger, may play an important role. Considerable effect was expended in investigating shifts in intercellular calcium of one particular cell line, Jurket, using flow cytometry methods. No changes in intercellular calcium were observed with this cell line under a wide variety of exposure conditions. It is not known at this time whether this is simply a cell type dependent phenomena or whether this is indeed an indication of a general negative response. It has been suggested that changes in calcium may be too small to be noted under these conditions.

Further studies (Ref. 7) suggest that the cell membrane is the necessary responsive agent. The effects of ELF on specific oncogene expression was apparent in intact Daudi cells. However, the same experiment run with intact nuclei only showed no response. Care was taken in this experiment to assure a parallel of the biological activity (see attached for details). This one would conclude that the membrane was cytoplasmic membrane was important for this assay.

Task 3. The objective of this task was to investigate specific therapeutic applications of ELF or ELF modulated RF fields as well as investigate any possible adverse effects for specific treatments. References 1, 2, 3 and 11 address this issue. First, We have found that 60 Hz fields can be useful in inhibiting the reabsorption of specific bone onlay bone graft to the facial skeleton. Not only was there inhibition of any resorption, but there was an actual increase in the size of the onlay under specific exposure conditions. These results indicate a clear biological response to 60 Hz magnetic fields of 1 Gauss or greater (Ref. 11). In addition, References 2 and 3 indicate

that by and large there are no detrimental effects from this exposure over the short period. However, there was an observation of a dose-dependent decrease of serum albumin and corresponding leakage of protein into the urine. This could suggest that the ELF field is interacting with the kidney glomerular basement membrane to effect the leakage of a specific protein. One rabbit did develop a tumor, and a cell line was isolated from this tumor to see if it was particularly ELF responsive. These results are reported in Ref 1. Although this cell line exhibited an interesting response to ELF, it would be impossible to associate the development of this one tumor with exposure to 60 Hz.

Again, these studies will hopefully add to the data base to provide further clues to the health effects of ELF and ELF modulated RF fields.

LIST OF PUBLICATIONS AND PRESENTATIONS

1. CHARACTERIZATION OF IMMORTALIZED CELL LINES ISOLATED FROM A SOFT TISSUE TUMOR DERIVED FROM A RABBIT SUBCHRONICALLY EXPOSED TO 60 HZ MAGNETIC FIELDS AT ONE GAUSS. J.T. Ning[1], G. Bushar[1], L. Li[1], S. Toyokuni[1], E.W. Czerska[1], D. Godar[1], E. Elson[2], C.C. Davis[3], L. Cress[1], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307, and [3]Dept. of Electrical Engineering, Univ. of Maryland, College Park, MD 20742. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992. Full paper to be published in Proceedings of Congress.

2. PATHOLOGICAL EVALUATION OF RABBITS SUBCHRONICALLY EXPOSED TO 60 HZ MAGNETIC FIELDS AT 1 GAUSS AND 5 GAUSS. S. Toyokuni[1], J.T. Ning[1], D. Thomas[1], E.W. Czerska[1], J. Casamento[1], C.C. Davis[2], E. Elson[3], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742, [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992. Full paper to be published in Proceedings of Congress.

3. DOSE-DEPENDENT HEALTH EFFECTS EXHIBITED IN RABBITS EXPOSED TO 60 HZ MAGNETIC FIELDS AT 1 GAUSS AND 5 GAUSS. J.T. Ning[1], E.W. Czerska1, J. Casamento[2], S. Toyokuni[1], C. St. Charles[2], F.E. Einsmann[2], B. Perry[2], E. Elson[3], C.C. Davis[4], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Clinical Laboratories, McGuire VA Medical Center, Richmond, VA 23249, [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307, [4]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992.

4. COMPARISON OF THE EFFECTS ON GENE EXPRESSION BY EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS EXPOSURE IN NORMAL AND TRANSFORMED HUMAN CELLS. M.L. Swicord[1], E.W. Czerska[1], J. Casamento[1], E. Elson[2], C.C. Davis[3], and J.T. Ning[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307, [3]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992.

5. EFFECTS OF CONTINUOUS AND PULSED 2450-MHz RADIATION ON SPONTANEOUS LYMPHOBLATOID TRANSFORMAITON OF HUMAN LYMPHOCYTES IN VITRO. Ewa M. Czerska[1], Edward C. Elson[3], Christopher C. Davis[2], Mays L. Swicord[1] and P. Czerski. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857 (EMC, MLS, PC), [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742 (CCD), [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307 (ECE). Bioelectormagneitcs 13:247-259 (1992)

6. THE EFFECTS OF ELF ON C-MYC ONCOGENE EXPRESSION IN NORMAL AND TRANSFOMRED HUMAN CELLS. E. Czerska[1], J. Casamento[1], C. Davis[2], E. Elson[3], J. Ning[1], and M. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857 (EC, JC, JN, MS), [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742 (CD), [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307 (EE). Proceedings of the Eighteeth IEEE Annual Northeast Bioengineering Conference, March 12-13, 1992. William J Ohley, ED.

7. COMPARISON OF THE EFFECTS OF EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELD EXPOSURE TO INTACT HUMAN CELLS OR THEIR NUCLEI WITHOUT CYTOPLASMIC MEMBRANE. J.T. Ning[1], H. Al-Barazi[1], J. Casamento[1], E.W. Czerska[1], C.C. Davis[2], E. Elson[3], L. Cress[1], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742, and [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992. Full paper to be published in Proceedings of Congress.

8. THE EFFECTS OF VARYING THE FREQUENCY AND MAGNETIC FIELD STRENGTH ON MORPHOLOGIC PHENOTYPE OF ELF RESPONSIVE MODEL CELL LINE. J.T. Ning[1], H. Al-Barazi[1], E.W. Czerska[1], J. Casamento[1], C.C. Davis[2], E. Elson[3], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742, [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992. Full paper to be published in Proceedings of Congress.

9. INTRACELLULAR CALCIUM SIGNALLING IN JURKAT E6-1 EXPOSED TO AN INDUCED ImV/cm, 60 Hz SINUSOIDAL ELECTRIC FIELD. Daniel B. Lyle[1], Janak Doshi[1], Thomas A. Fuchs[1], Jon P. Casamento[1], Yoshitatsu Sei[2], Prince K. Arora[2], and Mays L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857 (DBL, JD, TAF, JPC, MLS); [2]Laboratory of Neuroscience, NIDDK, National Institutes of Health, Bethesda, MD 20892 (YS, PKA). The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992.

10. PULSED ELECTROMAGNETIC FIELD EXPOSURE INTERFERES WITH ANTISENSE INHIBITION BOTH IN THE CYTOPLASMIC AND THE NUCLEAR COMPARTMENTS OF THE CELL. J.T. Ning[1], E.W. Czerska[1], E. Elson[2], C.C. Davis[3], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307, and [3]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992.

11. THE EFFECTS OF A SINGLE INTRAOPERATIVE IMMERSION IN VARIOUS CHEMICAL AGENTS AND ELECTROMAGNETIC FIELD EXPOSURE ON ONLAY BONE GRAFTS TO THE FACIAL SKELETON. J.T. Ning[1], E.W. Czerska[1], C.C. Davis[2], E. Elson[3], M.L. Swicord[1], and I.L. Wornom[4]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742, [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C., 20307, [4]Plastic and Reconstructive Surgery, Medical College of Virginia, Richmond, VA 23298. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992.

12. INTRACELLULAR CALCIUM SIGNALLING BY T-LYMPHOCYTES EXPOSED TO 60-HZ MAGNETIC FIELDS. Daniel B. Lyle[1], Lee A. Rosen[2], Thomas A. Fuchs[1], and Mays L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]National Institutes of Health, Bethesda, MD 20892. The First World Congress for Electricity and Magnetism in Biology and Medicine. June 14-19, 1992.

13. COMPARISON OF THE EFFECT OF ELF FIELDS ON TOTAL RNA CONTENT IN NORMAL AND TRANSFORMED HUMAN CELLS. John Ning[1], Heba Al-Barazi[1], Jon Casamento[1], Ewa Czerska[1], Christopher Davis[3], Edward Elson[2] and Mays Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307, and [3]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742. Accepted for publication in Ann. N.Y. Academy of Science.

14. COMPARISON OF THE EFFECT OF ELF FIELDS ON C-MYC ONCOGENE EXPRESSION IN NORMAL AND TRANSFORMED HUMAN CELLS.

Ewa Czerska[1], Heba Al-Barazi[1], Jon Casamento[1], Christopher Davis[3], Edward Elson[2], John Ning[1] and Mays Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307, and [3]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742.

Accepted for publication in Ann. N.Y. Academy of Science.

CHARACTERIZATION OF IMMORTALIZED CELL LINES ISOLATED FROM A SOFT TISSUE TUMOR DERIVED FROM A RABBIT SUBCHRONICALLY EXPOSED TO 60 HZ MAGNETIC FIELDS AT ONE GAUSS

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ABSTRACT

Several immortalized cell lines have been isolated from a tumor growth of a rabbit that was under going ELF exposure. One of these cell lines became anchorage independent in growth. Chracterization of this cell line revealed significant responses to ELF exposure. Further study of this cell line may lead to better understanding of mechanism of ELF interaction with the cell.

INTRODUCTION

A New Zealand White male rabbit which underwent whole body exposure to 60 Hz continuous waveform at 1 gauss for 6 hrs./day, 5 days/week developed a tumor mass approximately one month post initiation of the exposure regimen. At the end of the three months exposure period, the mass measured approximately 10 cm in diameter. In order to characterize and elucidate any relationship between 60 Hz magnetic field exposure and the development of this tumor growth, efforts were made to establish cell lines from explants of the tumor.

MATERIALS AND METHODS

At necropsy, the tumor was harvested immediately. Within 15 minutes of sacrifice, the tumor was minced in Hank's balanced salt solution without calcium or magnesium which had been prewarmed to 37°C in a sterile glass erlenmeyer flask supplemented with 1 mM EDTA. After mincing, the tissues were transferred to several glass erlenmeyer flasks containing prewarmed RPMI-1640 supplemented with 15% FBS, 25 mM HEPES, 1.25 U/ml Dispase, 0.5 mg/ml collagenase, 1 mg/ml hyaluronidase and incubated overnight at 37°C in a 5% CO_2 incubator. The next day, the media were removed from the erlenmeyer flasks and replaced with RPMI-1640 without serum but supplemented with 1.25 U/ml Dispase and 0.5 mg/ml of collagenase. After 30 min. incubation at 37°C, the suspension was pipetted vigorously and then centrifuged at low speed. The pellets were washed three times with RPMI-1640 + 20% FBS and

then seeded into several small tissue culture flasks. Cells were incubated in a CO_2 incubator at 37°C for 24 hours. After the cells appeared to have attached well to the substratum, the media were carefully pipetted off and replaced with fresh RPMI-1640 + 20% FBS and cells were then routinely split every 3 to 4 days to isolate immortalized cell lines. Eight of the 32 flasks inoculated with minced tumor explant proliferated and exhibited foci formation without reaching confluence on day 4 of culture.

The following analysis were used to characterize the immortalized cell lines: flow cytometry, electron microscopy, two-dimensional protein gel electrophoresis, and nuclear run-off assay.

RESULTS

The foci of these immortalized cell lines (Fig. 1A) were selectively passaged using a cloning cylinder to transfer focus forming cells. One of the resulting cell lines eventually exhibited anchorage independent growth. That cell line, R3, was further characterized by flow cytometry, electron microscopy, two dimensional protein electrophoresis and nuclear run-off assay. Flow cytometric analysis of RNA fluorescence with acridine orange exhibited significant increase in RNA fluorescence when exposed to ELF fields (Fig. 2). Another cell line (R4) which retained anchorage dependent growth did not exhibit any increase in RNA fluorescence with ELF exposure (Fig. 3). Electron microscopy study of R3 did not demonstrate any virus particle harbored by this cell line (Fig 4). Daudi, a human Burkitt lymphoma cell line which harbor the EB virus served as the positive control and showed viral particle (not shown). Two-dimensional protein gel electrophoresis revealed apparent induction of several new proteins in exposed cells (Fig. 5) as compared to control (Fig. 6). Nuclear run-off assay demonstrated over eight to eighteen fold increase in c-Ha-ras, N-ras and c-sis oncogene expression (Fig. 7, Table 1).

DISCUSSION

The immortalized cell lines isolated from the rabbit undergoing ELF exposure have now been continuously carried in culture for over 12 months and continue to exhibit foci formation (Fig. 1A) and continue to display uncontrolled proliferation potential. This is in contrast to primary cultures (Fig. 1B) isolated, using the same procedure, from normal soft tissues that were immediately adjacent to the tumor. In addition, these immortalized cell lines have been tested positive for anchorage independent growth. With 60 Hz, 1 gauss, exposure, these cells showed changes in 2-D protein gel pattern and significant increase in RNA fluorescence as analyzed by flow cytometry which are not exhibited by other immortalized cell lines.

There are three possible causes for the origin of the rabbit tumor. One possibility is spontaneous formation. Another possible cause may be due to endogenous oncogenic virus. This is unlikely since electron microscopy study did not identify any viral particles in this cell line. The third possible reason infer that somehow the origin of this tumor may be linked to ELF exposure. To test this possibility, time and efforts were spent in trying to isolate immortalized cell lines from the tumor. The observations that these rabbit tumor cell lines respond significantly to ELF fields when analyzed by multiple analytical techniques are supportive of this possible cause for the origin of the parent tumor. Currently, these anchorage independent cells have been inoculated into the flanks of nude mice in an attempt to assess their tumor forming potential with respect to ELF exposure.

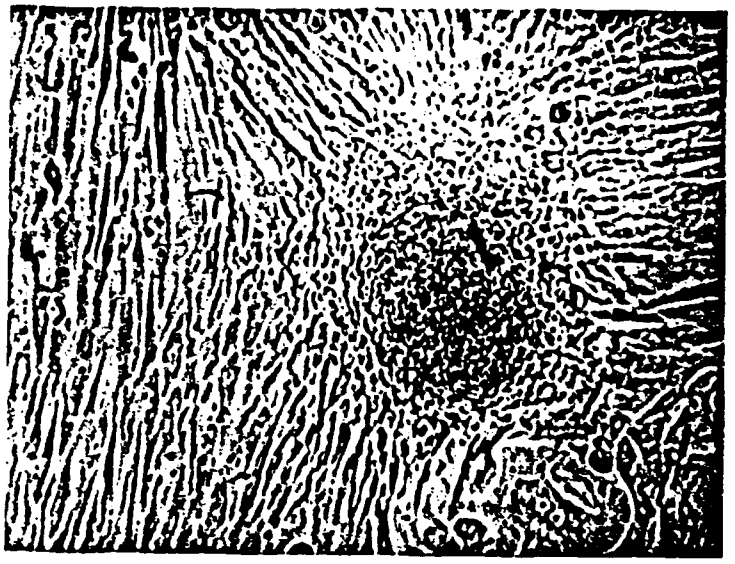

Fig. 1A. R3, focus forming cells.

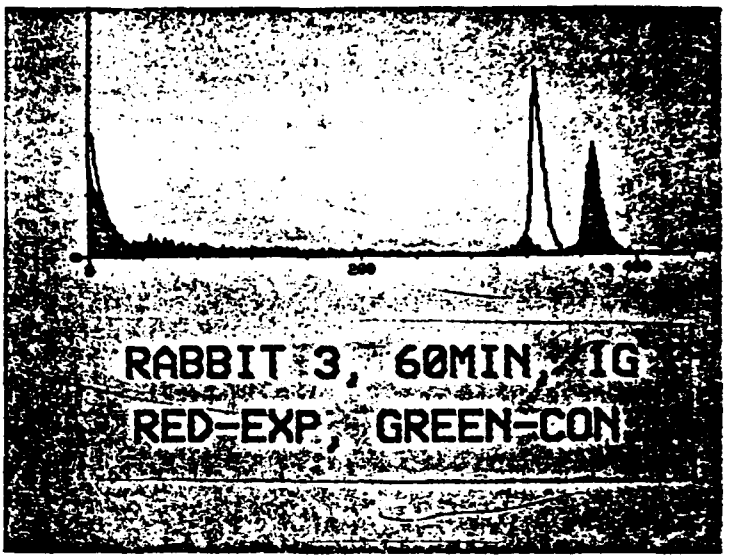

Fig. 2. R3, RNA fluorescence histograms of exposed and control cell populations. (Increase RNA result in fluorescence shift to the right on ordinate.)

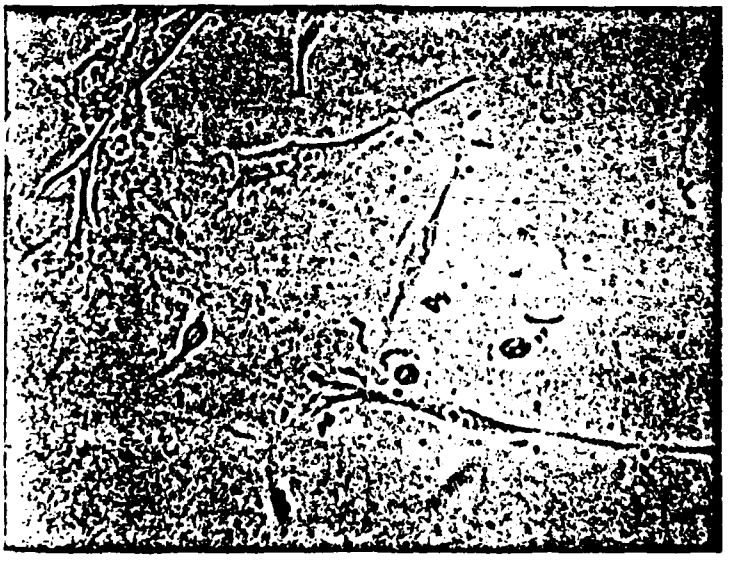

Fig. 1B. Primary culture isolated from surrounding normal tissue.

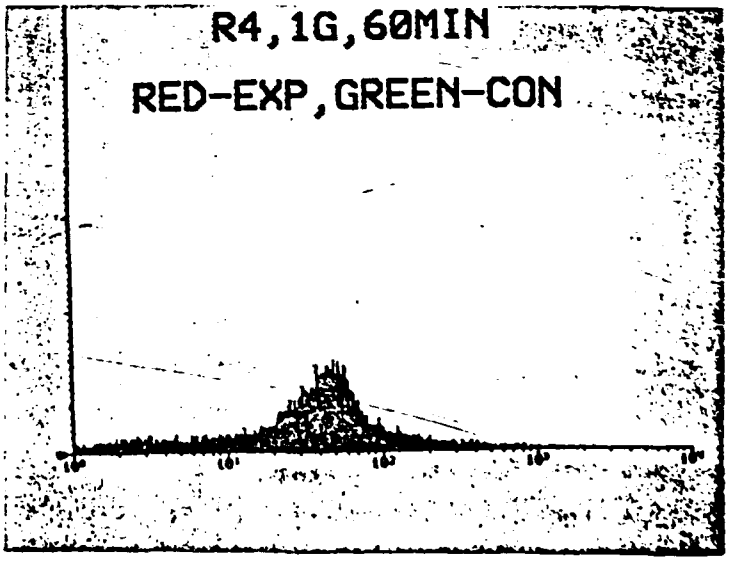

Fig. 3. R4, RNA fluorescence histograms of exposed and control cell population.

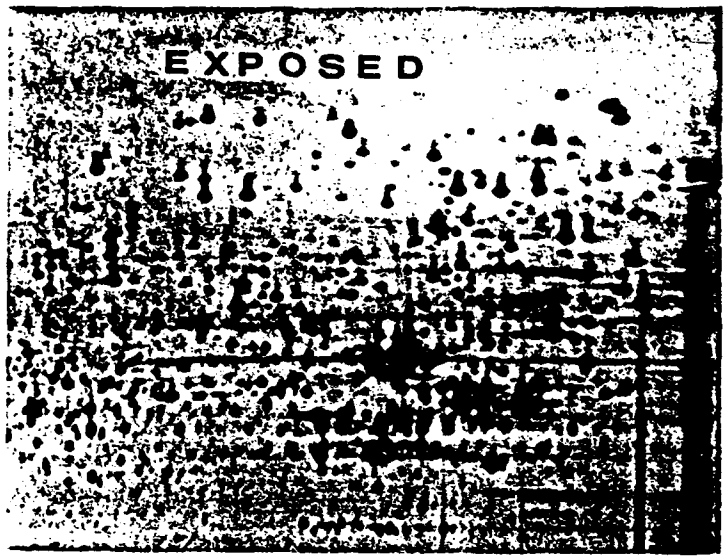

Fig. 5. Two-dimensional protein gel of R3 exposed to ELF. (Arrows indicate new protein spots.)

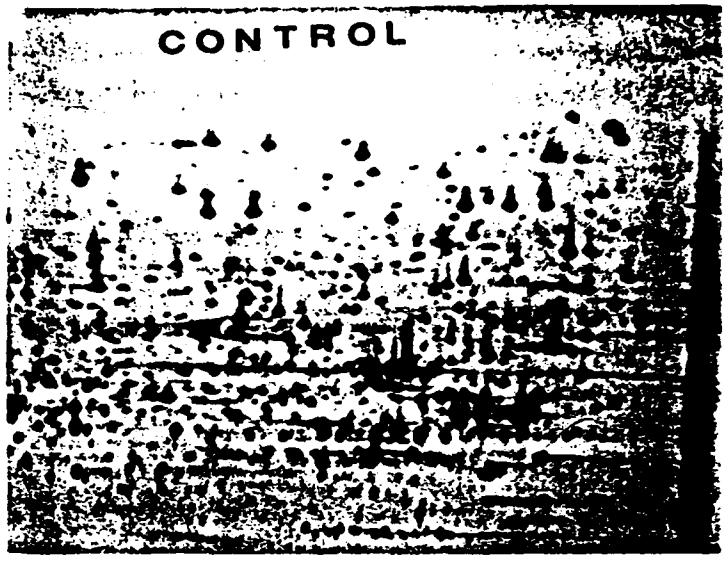

Fig. 6. Two-dimensional protein gel of R3 control.

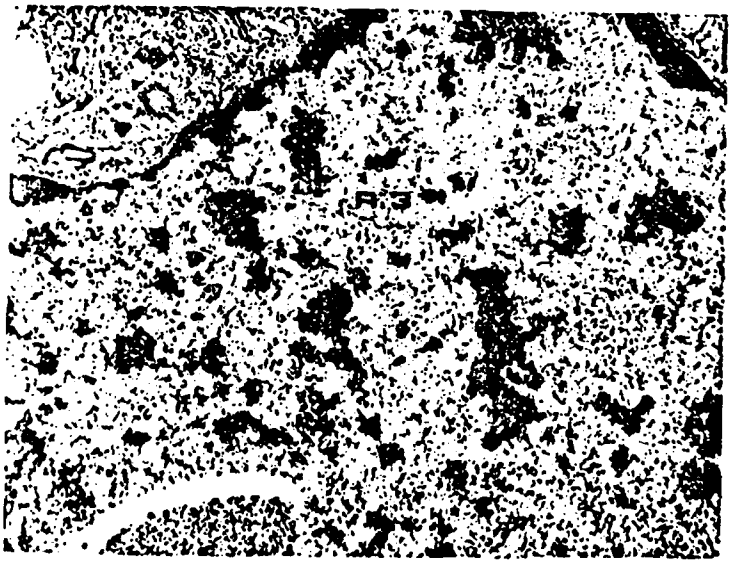

Fig. 4. Electron micrograph of R3 cell.

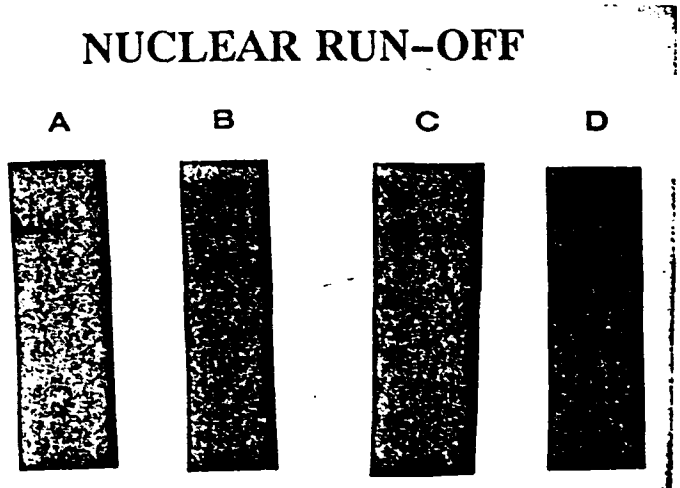

Fig. 7. Nuclear run-off assay.
A) R3 exposed
B) R3 control
C) R3 nuclei exposed
D) R3 nuclei control.

PATHOLOGICAL EVALUATION OF RABBITS SUBCHRONICALLY EXPOSED TO 60 HZ MAGNETIC FIELDS AT 1 GAUSS AND 5 GAUSS

J.T. Ning[1], S. Toyokuni[1], D. Thomas[1], E.M. Czerska[1], J. Casamento[1], C.C. Davis[2], E. Elson[3], and M.L. Swicord[1]. [1]Center for Devices and Radiological Health, Food and Drug Administration, Rockville, MD 20857, [2]Dept. of Electrical Engineering, University of Maryland, College Park, MD 20742, [3]Dept. of Microwave Research, Walter Reed Army Institute of Research, Washington, D.C. 20307.

ABSTRACT

Numerous *in vitro* and *in vivo* biological effects due to exposure to ELF fields have been reported (1). However, there is a paucity of data relating those findings to health effects. This prompted us to initiate a pilot animal study which would address possible disease pathology. Twelve young adult 3 kg New Zealand White male rabbits were divided into three groups. One group served as the control and was exposed to ambient magnetic fields which ranged from 1 – 8 mG. The second and third groups were exposed to 1 and 5 gauss 60 Hz magnetic fields, respectively, for 6 hours/day, 5 days/week for the 14 week period. The animals were examined visually throughout the entire subchronic 14 week period. One rabbit which was exposed to the 1 gauss magnetic field developed a tumor growth on the left posterior flank 1 month into the exposure regimen. At the end of 14 weeks, Complete autopsies were performed and specimens from the heart, lungs, spleen, adrenal glands, kidneys, liver, gallbladder, pancreas, stomach, jejunum, ileum, large intestine, urinary bladder, testis, submandibular and sublingual glands, femur, quadriceps femoris muscle, cerebrum (cortex and hippocampus), cerebellum, and retina as well as any tumor or enlarged lymph nodes were obtained. Each specimen was fixed with 2.5% paraformaldehyde, embedded in paraffin and sectioned at 4 μm. Hematoxylin & Eosin stain, Pearls' iron stain and reticulin stain were used. There were no positive findings in all the organ specimens with the exception of the lung, liver and urinary tract. In the hepatocytes of exposed animals, lipofuscin deposition was more remarkable. Slight hemosiderin deposition in hepatocytes of control animals was less prominent in the exposed animals. One of the control rabbits showed an abscess in the lung, likely to be of bacterial origin. All the exposed rabbits revealed cystitis with erosion, squamous metaplasia, lymphocyte and plasma cell infiltrations in the bladder mucosa indicating chronic inflammation. One of the exposed rabbits showed chronic

inflammation in the renal pelvis. The tumor in the flank was diagnosed as an abscess. However, this pilot animal study revealed no significant disease pathology associated with subchronic exposure of 60 Hz magnetic fields at 1 and 5 gauss.

INTRODUCTION

Extremely low frequency (ELF) electromagnetic fields (EMFs) have been reported to cause various biological effects (1). Increasingly, ELF medical devices are being tested under clinical trials for potential therapeutic applications in clinical medicine. Concurrent with the optimism of new modalities of treatment, there is also concern for possible adverse effects on the human patients. That concern is heightened by recent reports in the literature on the increased risk of cancer, especially leukemia and certain central nervous system cancers, being linked to occupational EMF exposure (2–4). Even though ELF devices are currently being used clinically to treat non-union fractures, only limited numbers of studies have been done on animal models with regard to health effects. This led us to initiate this subchronic study which assesses possible disease pathology associated with 60 Hz ELF magnetic field exposure at one and five gauss.

MATERIALS AND METHODS

Twelve young adult 3 kg New Zealand White male rabbits were divided into three groups. One group served as control and was exposed to ambient EMF in the Twinbrook Research Laboratory animal care facility. The second and third group were exposed to 1 and 5 gauss ELF fields at 60 Hz, respectively, for 6 hr/day, 5 days/week for 14 weeks. Each day, the animals to be exposed were placed in plastic cages which were then placed in a large Helmholtz coil exposure system. All rabbits were housed in the same room in the animal care facility and received the same food and water *ad lib*. The temperature, humidity and lighting were controlled and monitored throughout the experiment. The rabbits were examined daily for any observable

gross changes. After 14 weeks of exposure, the rabbits were humanely sacrificed and necropsy was performed. Specimens from the heart, lung, spleen, adrenal gland, kidney, liver, gallbladder, pancreas, stomach, jejunum, ileum, colon, urinary bladder, testis, submandibular and sublingual gland, femur, quadriceps femoris muscle, cerebrum (cortex and hippocampus), cerebellum, and retina as well as any tumor or enlarged lymph nodes were obtained. Paraffin embedded histologic sections were prepared and stained with Hematoxylin & Eosin. For liver and kidney specimens, Pearls' iron stain and reticulin stain were also used. In addition, kidney specimen were processed for transmission electron microscopy (TEM).

RESULTS

Examination of the H&E-stained tissue sections for morphology under light microscopy revealed no abnormal findings in all the exposed rabbits' organs with the exception of the lung, liver and urinary tract. In the hepatocytes of the ELF exposed rabbits, lipofuscin granules were more prominent than control animals (Fig.1a-c), and deposition was observed mostly in the periportal area. In the hepatocytes of control rabbits, slight deposition of hemosiderin was observed around the central vein, but in those of exposed rabbits, hemosiderin deposition was less prominent. One of the control rabbit showed abscess in the lung, likely to be of bacterial origin. All the exposed rabbits had mild to severe cystitis with erosion, squamous metaplasia, lymphocyte and plasma cell infiltrations in the bladder mucosa indicating chronic inflammation. However, in the control group, only one rabbit showed chronic cystitis. One of the exposed rabbits showed chronic inflammation at renal pelvis. Reticulin stain revealed no abnormality in the glomeruli of the exposed animals. TEM showed normal appearing basement membrane in the glomeruli of control and exposed kidneys without abnormal deposition or fusion of the podocytes.

Examination of the tumor from the rabbit exposed to 60 Hz ELF fields at one gauss

showed a non-indurated cystic soft tissue mass in the left posterior flank. The tumor was movable by palpation. When first noticed after 1 month of exposure, the tumor measured 4 cm in diameter. The tumor steadily increased in size to approximately 10 cm in diameter at necropsy. During necropsy, a yellowish amorphous semiliquid content was observed after opening the tumor sac. Pathological Examination under light microscopy showed abscess with chronic inflammation (Fig.2). In some part of the abscess wall, fibroblasts with slight atypia were observed (Fig.3). No evidence of neoplasm was found. Several different portions of the abscess wall were inoculated in tissue culture media in an attempt to isolate possible transformed cell lines. Isolation and characterization of cell lines derived from the abscess wall will be presented in an companion paper.

DISCUSSION

Histological examinations revealed no major changes in morphology except prominent lipofuscin deposition in the liver and urinary tract infection in the exposed animals. Lipofuscin represents the indigestible residues of autophagic vacuoles formed during aging or atrophy, and its derivation through lipid peroxidation of polyunsaturated lipids of subcellular membranes are suggested (5). Whether chronic exposure of ELF leads to increased cellular lipid peroxidation will be an interesting area for further study. Hemosiderin is an iron-protein complex storing excess iron in the cytoplasm (5). Iron kinetics may be changed by exposure to ELF fields to effect decreased storage of iron. Since excess iron appears to be associated with carcinogenesis (6), further evaluation of the effect of ELF on iron metabolism is warranted.

We examined the kidneys both by light microscopy and electron microscopy in order to clarify the possible role of kidney in decreased serum albumin and increased urinary protein described in a companion paper. These studies showed normal structure of endothelium, basement

membrane and podocytes. Thus, the kidney is not responsible for the altered protein patterns in the serum and urine provided that protein leakage is always associated with morphological changes detectable by electron microscopy. One of the possible explanations for protein alteration would be immunosuppression since all the exposed animals showed chronic urinary tract infection. This idea may explain serum albumin decrease, urinary protein increase and high frequency of urinary tract infection. Further investigation is necessary for the evaluation of immunological factors such as T cell, B cell, polymorphonuclear leukocyte (PMN) and macrophage function.

The tumor found on the hip of one of the exposed rabbits was identified as a non-neoplastic abscess. Several cell lines resembling fibroblast in phenotype were established from cells isolated from the abscess wall. Surprisingly, no bacterial contamination was observed. One cell line became anchorage independent and dramatically responded to further ELF exposure. (See the companion paper for details). Since we found fibroblasts in a local area of the abscess wall that was slightly atypical, those atypical cells may be the origin of our cell lines. Why abscess was formed on the hip is unknown. We observed abundant PMN in the tumor wall and cavity. The reactive oxygen species produced by PMN or macrophage (7) may be responsible for the initiation of fibroblasts.

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FIGURE LEGENDS

Fig.1. a, control rabbit, liver. Hepatocytes show no cytoplasmic granules (H&E, x 400); b, 60 Hz, 1 gauss–exposed rabbit, liver. Hepatocytes at the periportal area show cytoplasmic brown pigment deposition (H&E, x 400); c, same as b. Negative results by Pearls' iron staining indicate that these intracytoplasmic granules are lipofuscin (Pearls' iron stain, x 400).

Fig.2. Wall of the tumor that appeared in 60 Hz, 1 gauss–exposed rabbit. Diagnosis, abscess wall. Erosion, infiltration of polymorphonuclear leukocytes and macrophages are prominent (H&E, x 400).

Fig.3. Abscess wall. Fibroblasts with slight atypia were seen in a small area of abscess wall (H&E, x 400).

FIG. 1a

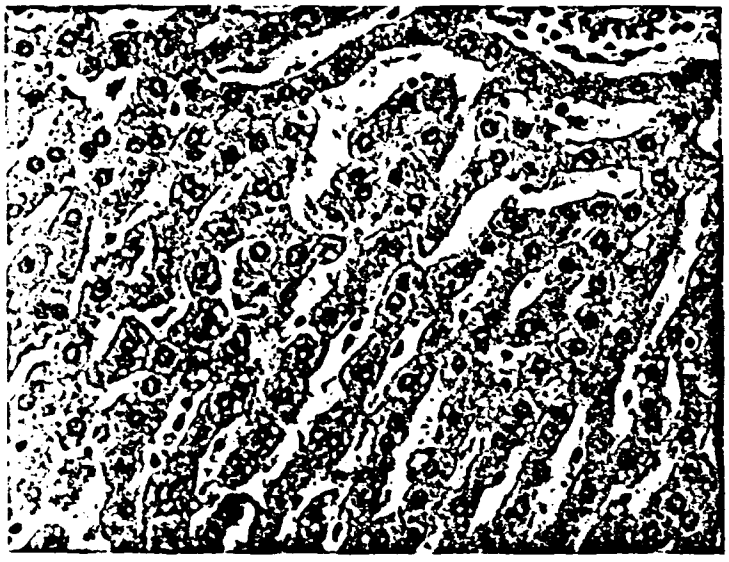


FIG. 1b

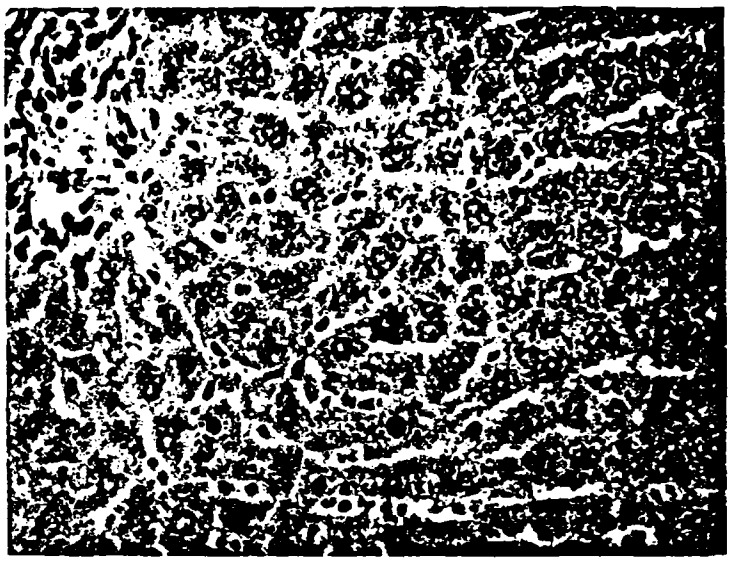


FIG. 1c

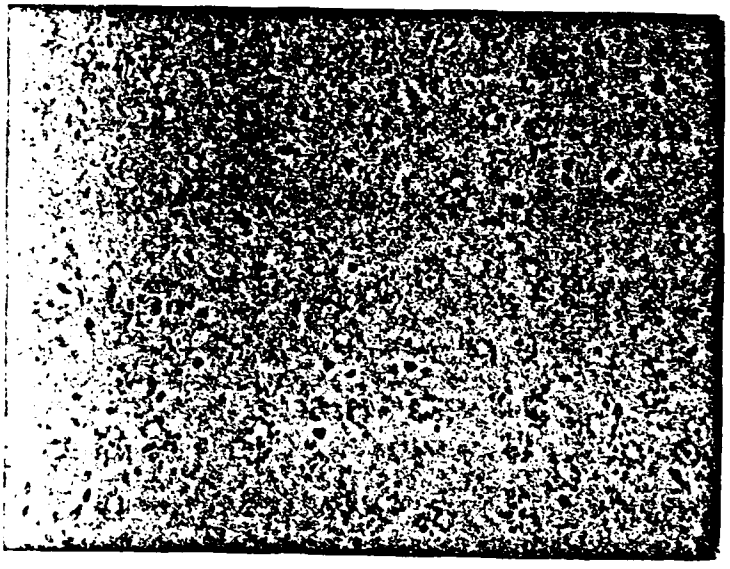


FIG. 2

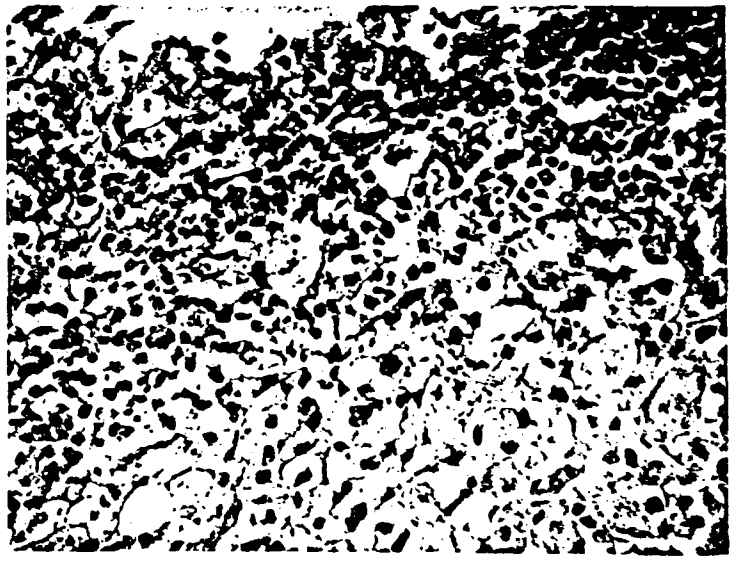
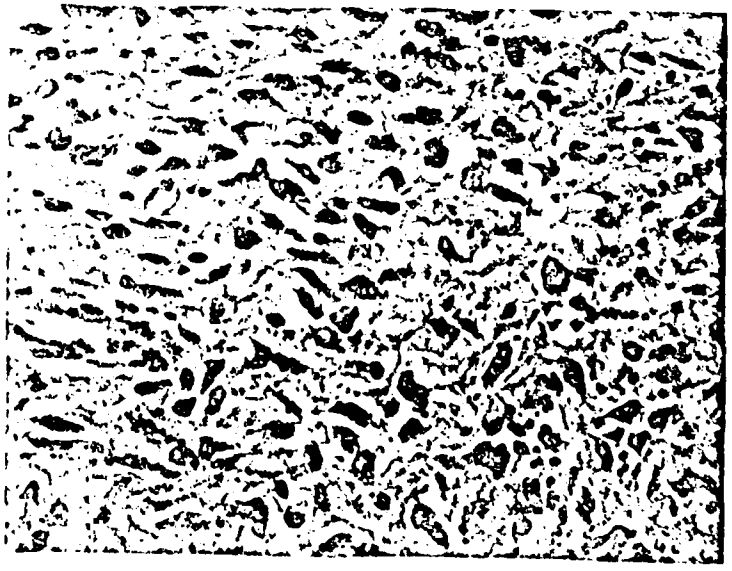


FIG. 3



DOSE-DEPENDENT HEALTH EFFECTS EXHIBITED IN RABBITS EXPOSED TO 60 HZ MAGNETIC FIELDS AT 1 GAUSS AND 5 GAUSS

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ABSTRACT

Recent epidemiological studies have reported a possible association between presumed exposure to 60 Hz magnetic fields and health effects of persons working with power generation and transmission equipment. For a recent review, please see Coleman and Beral, Int. J. Epidemiol. 17:1–13, 1988. We have initiated a pilot animal study to assess possible relationships between 60 Hz magnetic field exposure and any observable health effect. Twelve 3 kg New Zealand White male rabbits were divided into three groups. One group served as a control and was exposed to ambient Background magnetic fields ranged from 1 – 8 mG. The second and the third group were exposed to 1 and 5 gauss magnetic fields respectively for 6 hours/day, 5 days/week for the 14 week period. One rabbit which was exposed to 1 gauss magnetic field developed a tumor growth on the left posterior flank 1 month into the exposure regimen. At the end of 14 weeks complete autopsies were performed and specimen from all major organ systems were obtained for histopathologic studies. Blood and urine specimen were obtained by cardiac and suprapubic punctures, respectively, prior to sacrifice. Coded blood and urine specimen were analysed by an independent clinical laboratory blinded to the study. Analysis of the data indicates that blood albumin decreased while urine proteins increased with increasing magnetic field exposure. Urine protein electrophoresis performed also exhibited a dose dependent accumulation of a single protein band (migrating in the alpha 2 region of human serum standard) with increasing exposure. These trends suggest that 60 Hz magnetic field may somehow interact with the kidney glomerular basement membrane to produce the dose-dependent health effects observed in this study. The appearance of this single unidentified protein band with ELF exposure in the urine may serve as a convenient marker for monitoring extremely low frequency (ELF) exposure.

INTRODUCTION

Many reports have been published in the literature suggesting chronic health effects with electromagnetic field (EMF) exposure (1,2). Animal studies with mice have found increased body weight gain, increased organ weight and elevation of certain circulatory hormones with exposure to extremely low frequency (ELF) EMFs (3–5). In vivo studies on rabbits showed transient delay in growth when exposed outdoors. But, the results were not reproduced indoors with 10 kV/m electric fields (6). Therefore, a pilot study to assess 60 Hz magnetic fields at one gauss and five gauss were initiated to search for any health effects detectable in the rabbit that may be linked to ELF exposure.

MATERIALS AND METHODS

Twelve 3 kg New Zealand White male rabbits were divided into three groups. One group served as control and was exposed to ambient EMF in the Twinbrook Research Laboratory animal care facility. The second and third group were exposed to 1 and 5 gauss ELF at 60 Hz respectively for 6 hr/day, 5 days/week for 14 weeks. Each day, the animals to be exposed were placed in plastic cages which were then placed in a large Helmholtz coil exposure system. All rabbits were housed in the same room in the animal care facility and received the same food and water ad lib. The temperature, humidity and lighting were controlled and monitored throughout the experiment. The rabbits were examined daily for any observable gross changes. After 14 weeks of exposure, the rabbits were humanely sacrificed and necropsy was performed. Histological specimen from the following organs were obtained: Heart, lung, spleen, adrenal gland, kidney, liver, pancreas, stomach, jejunum, ileum, colon, urinary bladder, testis, quadricep femoris muscle, femur, and submandibular gland. Paraffin embedded slides were prepared and stained for each specimen. Serum and urine specimen were also obtained. The following clinical

laboratory tests were performed on coded specimen: Complete Blood Count (CBC), Chemistry Profile, urine analysis, serum and urine electrophoresis.

RESULTS

Table 1 shows the effect of 14 weeks exposure on the body weight of the rabbits to 1 gauss and 5 gauss at 60 Hz. There are no significant differences in body weight between control and ELF exposed rabbits. Table 2 shows a comparison of organ weights of each rabbit adjusted for total body weight. There is an apparent 20% increase in lung weight of rabbits exposed to 5 gauss ELF. There were no significant differences found in CBC or chemistry profile except dose dependent decreases of albumin concentration in the serum. Concurrently, there are dose-dependent leakage of proteins into the urine. These results are shown in Table 3 and 4 respectively. Because of the positive correlation between both serum albumin and total protein levels in the urine and the magnetic field strength, serum and urine protein electrophoresis were performed. The electrophoretograms are shown in Figures 1 and 2 respectively. Figures 3 and 4 depict the scan of control and 1 gauss ELF exposed serum protein electrophoresis pattern respectively. There appears to be an additional migrating band in Figure 1 in two of the four rabbit serum exposed to ELF as compared to control. This is illustrated in the scan profiles of Figure 3 and 4. Figure 2 also shows a new protein band in the urine of exposed rabbits which co-migrate with alpha 2 region of human serum standard. Control rabbit urine was devoid of this protein band. This new band is barely visible in the urine samples from rabbits exposed to 1 gauss ELF. The same migrating band is clearly visible in the urine samples from rabbits exposed to 5 gauss ELF.

The histopathological evaluation of each of the organs and the tumor found in one of the rabbits exposed to 1 gauss ELF will be reported in a companion paper.

DISCUSSION

Unlike the reports in the literature, this pilot study did not show increases in total body weight nor any organ specific weights. The elevation of lung weight in rabbits exposed to 5 gauss ELF were probably due to pooling of blood into the lung resulting in congestion during sacrifice. Instead, this study demonstrated positive effects of subchronic exposure of 60 Hz ELF on serum albumin and urine protein. It is interesting that the perturbations observed correlate with ELF field strength. We observed a dose-dependent decreases of serum albumin and corresponding leakage of protein into the urine. One possible explanation is that perhaps ELF is interacting with the kidney glomerular basement membrane to effect the leakage of this specific protein into the urine with ELF exposure. When analyzed by urine protein electrophoresis, surprisingly, there is a dose-dependent appearance of a protein band co-migrating to the alpha 2 region of the human serum standard. The identity of this new protein band needs to be elucidated and it is unlikely to be albumin by its electrophoretic pattern of migration. The appearance of this urine protein band may serve as a convenient marker for ELF exposure if it is reproducible. It would be of interest to investigate whether this phenomenon can also be found in primates and humans.

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TABLE 1. EFFECT OF SUBCHRONIC ELF EXPOSURE
BODY WEIGHT (gram)

	MEAN	S.D.
CONTROL	3452	354
ELF, 1G	2798	375
ELF, 5G	2988	306

(n=4)

TABLE 2. EFFECT OF SUBCHRONIC ELF EXPOSURE
SPECIFIC ORGAN WEIGHT*

ORGAN	CONTROL Mean	S.D.	ELF, 1G Mean	S.D.	ELF, 5G Mean	S.D.
BRAIN	0.30	0	0.38	0.05	0.32	0.05
HEART	0.20	0	0.22	0.05	0.22	0.05
KIDNEY	0.28	0.05	0.30	0	0.30	0
LUNG	0.35	0.06	0.38	0.05	0.48	0.05
LIVER	1.80	0.24	2.28	0.28	2.18	0.22
TESTIS	0.12	0.05	0.18	0.05	0.18	0.05

EXPRESSED AS % OF BODY WEIGHT (n=4)

TABLE 3. EFFECT OF SUBCHRONIC ELF EXPOSURE
SERUM ALBUMIN (g/dl)

	Mean	SEM
CONTROL	4.0	0.3
ELF, 1G	3.4	0.2
ELF, 5G	3.1	0.3

1-4)

TABLE 4. EFFECT OF SUBCHRONIC ELF EXPOSU
URINE TOTAL PROTEIN

	TOTAL PROTEIN
CONTROL	NEG
ELF, 1G	TRACE - 30MG
ELF, 5G	30MG - 300MG

(n=4)

COMPARISON OF THE EFFECTS ON GENE EXPRESSION BY EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS EXPOSURE IN NORMAL AND TRANSFORMED HUMAN CELLS

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ABSTRACT

Many *in vitro* literature studies have reported that extremely low frequency (ELF) electromagnetic field exposure may have varying effects on gene expression in different human cell lines. To more precisely characterize the effect of ELF exposure on gene expression in vitro, we have investigated the use of laser flow cytometry. Laser flow cytometric analysis has the advantage that a large population of intact cells can be acquired and analyzed easily and the relative quantities of a variety of cellular constituents can be measured simultaneously with high accuracy and precision. HL-60, a human promyelogenous leukemia cell line, Daudi, a human lymphoma cell line with translocated c-myc oncogene and GM1500B, a human lymphoma cell line with intact c-myc oncogene were employed in this study. The cells were exposed for 30 min. and 60 min. to either 60 Hz continuous waveform at 1 gauss or pulsed electromagnetic fields (PEMF) generated by a Biosteogen Model CU-204 generator (Electro-Biology, Inc.). Immediately post-exposure, the cells were fixed in 50% ice-cold ethanol and processed for flow cytometry using acridine orange as the fluorochrome. Total RNA fluorescence increased in Daudi lymphoma cells that were exposed to ELF while exposure to ELF exhibited either no appreciable effects on RNA fluorescence or decreased RNA flouuresccnce with exposure in HL-60, GM 1500B or stimulated peripheral human lymphocytes.

INTRODUCTION

Extremely low frequency electromagnetic field exposure can have different effects on gene transcription in vitro in human cells (1-3). Work published previously by the authors showed differential effects of ELF fields on c-myc oncogene expression in HL-60, a promyelogenous leukemia cell line and Daudi, a Burkitt lymphoma cell line (4). To analyze with more precision, the transcriptional effects of ELF field exposure in vitro, we investigated the

application of laser flow cytometry. Flow cytometric analysis has the advantage that a large population of intact cells can be acquired and analyzed easily and the relative quantities of a variety of cellular constituents can be simultaneously measured with high precision and accuracy. Therefore, we investigated the effect of ELF fields on cellular transcription by flow cytometry on intact HL-60 and Daudi cells. The cell line GM 1500B, a human lymphoblastoid cell line similar to Daudi except it contains the intact c-myc oncogene was also assessed as well as sttimulated peripheral human lymphocytes.

MATERIALS AND METHODS

1. CELL CULTURES

HL-60, a human promyelygenous leukemia cell line, Daudi, a human lymphoma cell line with translocated c-myc and GM 1500B another human lymphoma cell line containing intact c-myc oncogene were routinely cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum. Normal peripheral human lymphoctes were routinely cultured in chromosome medium containing 2.5 ug/ml Phytohemagglutinin (PHA).

2. EXPOSURE PROTOCOL

Approximately 5 X 10^5 cells in suspension were placed in Corning T75 tissue culture flask containing 10 ml fresh medium one hour prior to exposure. After preincubation, the cells were exposed for 30 min. and 60 min. in a 5% CO_2 incubator at 37^{0}C to both 60Hz magnetic fields at 1 gauss and pulsed electroamgnetic fields (PEMF) generated by a Biosteogen Model CU-204 generator (Electro-Biology, Inc.). Immediately post exposure, the cells were fixed in 50% ice-cold ethanol.

3. FLOW CYTOMETRY

The fixed cells were washed twice in PBS + 10% human serum. 0.2 ml of the cell

suspension was subsequently permeabilized with 0.4 ml of ice-cold solution containing 0.1% triton x-100, 0.05N HCl and 0.15N NaCl. The suspensions were mixed gently and 1.2 ml of acridine orange (6 ug/ml) in phosphate-citrate buffer were added to the suspension. After 5 minutes incubation at room temperature, the stained cells were filtered through a 37 micron mesh and analyzed for RNA fluorescence using a Beckton-Dickinson FACScan flow cytometer. For each histogram, 10,000 cells were acquired and analyzed.

4. NORTHERN ANALYSIS

Post-exposure, total RNA was immediately extracted with guanidinium isothiocyanate and and the RNA was pelleted through a CsCl step gradient ultracentrifugation. The purified total RNA was then run in a denaturing formaldehyde agarose gel and transferred to polymer membrane. Nick-translated human c-myc exon 3 probe and a beta-actin probe were used to hybridize against the Northern blots. Washing was carried out under stringent conditions and autoradiography was carried out at -70°C for 2-3 days.

RESULTS

Daudi, the human lymphoma cell line with translocated c-myc oncogene exhibited increased RNA fluorescence when exposed to both 60 Hz magnetic fields at 1 gauss and 15 Hz pulse train (Fig. 1). PEMF induced larger shifts of RNA fluorescence than 60 Hz fields. HL-60 (Fig. 2), peripheral human lymphocyte (Fig. 3) and GM 1500B (Fig. 4), a human lymphoblastoid cell line with intact c-myc oncogene exhibited variable decrease or no response to ELF fields.

Northern analysis of Daudi (Table 1), HL-60 (Table 2) and GM 1500B (Table 3) with c-myc probe exhibited the same trend as flow cytometry. Daudi with translocated c-myc showed slight activation of c-myc oncogene expression when exposed to 60 Hz ELF field at 1 gauss. GM 1500B, with intact c-myc oncogene, and HL-60 exhibited either no change or a decrease

in c-myc oncogene expression.

DISCUSSION

The metachromatic fluorochrome acridine orange (AO) can differentially stain DNA and RNA (5). The use of this fluorochrome in flow cytometric analysis enabled us to accurately observe the cellular RNA content. Since cellular RNA content is closely associated with cell proliferation (6), flow cytometry allows us to accurately assess the often weak effect of ELF fields on cellular proliferation in Daudi, GM 1500B, HL-60 and stimulated peripheral human lymphocytes. By overlaying RNA histograms of exposed and control cell populations in different colors, any change in RNA fluorescence can be easily visualized between exposed and control cell populations.

Flow cytometry with AO revealed that Daudi responded to ELF exposure by increasing RNA fluorescence, whereas, HL-60 and human peripheral lymphocytes exhibited no change or decreased fluorescence. This led us to hypothesize that the differential responses shown by Daudi and HL-60 to ELF exposure may be due to Daudi expressing the translocated c-myc oncogene (7) and HL-60 expressing the intact c-myc oncogene. In Burkitt's lymphoma, the c-myc oncogene located on the long arm of chromosome 8 is translocated to a region upstream of an immunoglobulin constant region gene. In the new translocated location, the c-myc oncogene is actively transcribed and may have escaped its normal control mechanism (8). Thus, translocation may lead to the deregulation of the c-myc oncogene in Daudi. It is possible that ELF fields may have an effect on the deregulated c-myc oncogene in Daudi but under normal regulation, may not have an effect on the intact c-myc oncogene.

To test this hypothesis, we measured total RNA fluorescence and performed Northern analysis on a human lymphoblastoid cell line, GM 1500B which contain the intact c-myc

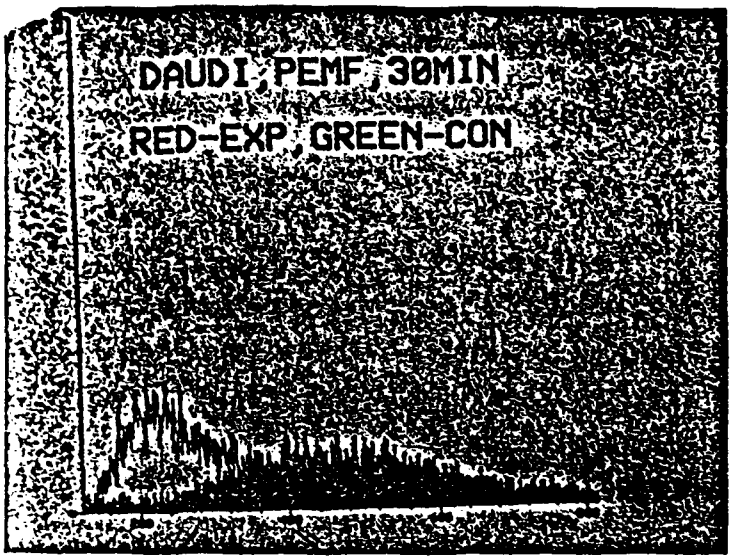

Fig. 1. RNA histograms of Daudi cells Exposed to PEMF for 30 min.

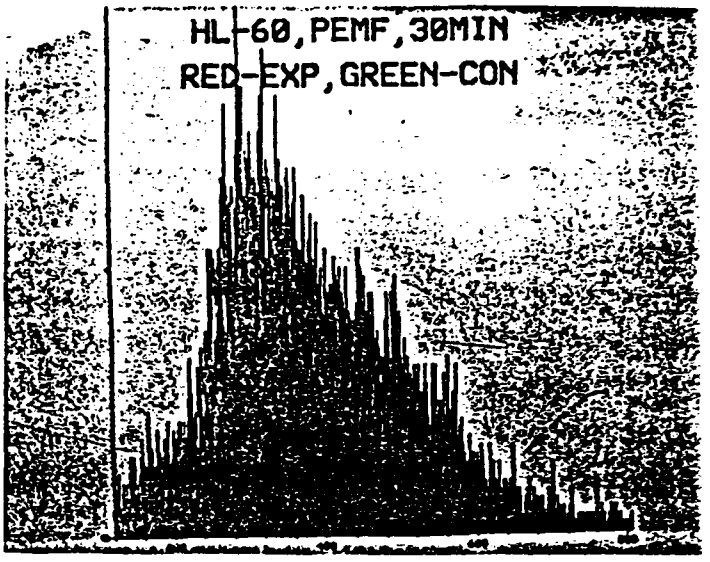

Fig. 2. RNA histograms of HL-60 cells exposed to PEMF for 30 min.

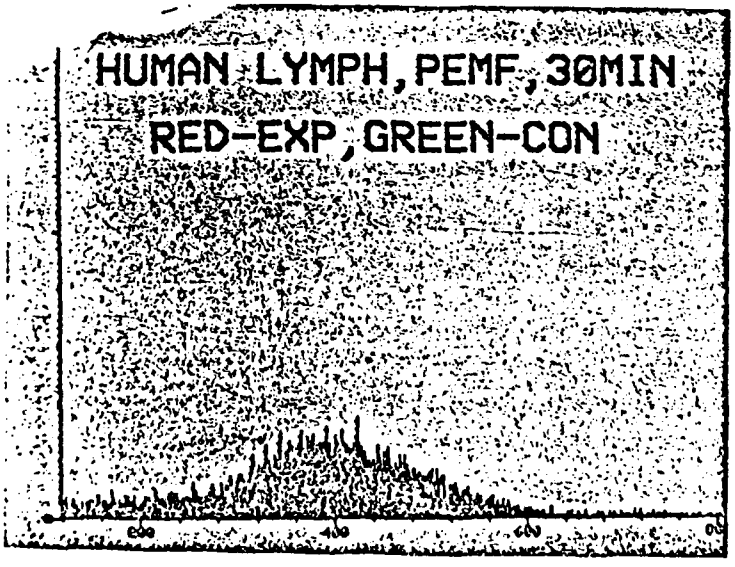

Fig. 3. RNA histograms of human lymphocytes exposed to PEMF for 30 min.

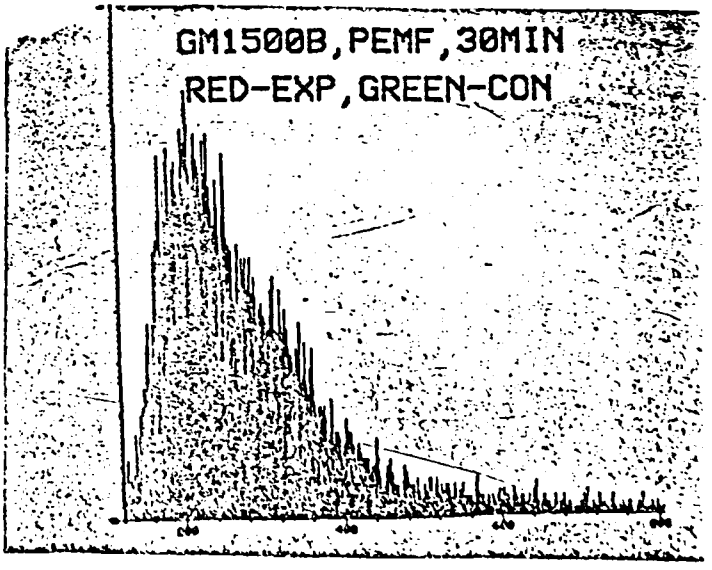

Fig. 4. RNA histograms of GM 1500B cells exposed to PEMF for 30 min.

Bioelectromagnetics 13:247–259 (1992)

Effects of Continuous and Pulsed 2450-MHz Radiation on Spontaneous Lymphoblastoid Transformation of Human Lymphocytes In Vitro

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Normal human lymphocytes were isolated from the peripheral blood of healthy donors. One-ml samples containing (10^6) cells in chromosome medium 1A were exposed for 5 days to conventional heating or to continuous wave (CW) or pulsed wave (PW) 2450-MHz radiation at non-heating (37°C) and various heating levels (temperature increases of 0.5, 1.0, 1.5, and 2 °C). The pulsed exposures involved 1-μs pulses at pulse repetition frequencies from 100 to 1,000 pulses per second at the same average SAR levels as the CW exposures. Actual average SARs ranged to 12.3 W/kg. Following termination of the incubation period, spontaneous lymphoblastoid transformation was determined with an image analysis system. The results were compared among each of the experimental conditions and with sham-exposed cultures. At non-heating levels, CW exposure did not affect transformation. At heating levels both conventional and CW heating enhanced transformation to the same extent and correlate with the increases in incubation temperature. PW exposure enhanced transformation at non-heating levels. This finding is significant ($P < .002$). At heating levels PW exposure enhanced transformation to a greater extent than did conventional or CW heating. This finding is significant at the .02 level. We conclude that PW 2450-MHz radiation acts differently on the process of lymphoblastoid transformation in vitro compared with CW 2450-MHz radiation at the same average SARs.

Key words: 2450-MHz microwaves, pulsed and continuous waves, thermal effects

INTRODUCTION

Stodolnik-Baranska [1967] reported that 15-min or 4-h exposures to 2,990-MHz pulsed microwaves repeated daily over 3 or 5 days enhanced spontaneous

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This study is dedicated to our beloved colleague, Przemyslaw Czerski, who died on April 15, 1990.

transformation of human lymphocytes in vitro. Enhancement of transformation was also observed in phytohemagglutinin (PHA) stimulated lymphocytes exposed during the last 6 hours of a 72-h incubation period [Stodolnik-Baranaska, 1974]. Roberts et al. [1983] exposed PHA-stimulated or nonstimulated human lymphocytes for 2 h to 2450-MHz continuous waves and subsequently examined lymphoblastoid transformation following incubation for 3 days (72 h). They did not find differences between exposed and sham-exposed cultures. These authors concluded that their results indicated that microwave exposed human lymphocytes may respond normally to a second biological factor, such as antigens or infections, and thus did not confirm Stodolnik-Baranska's [1967, 1974] findings.

This study makes an attempt to resolve the discrepancy between these findings. There are many plausible explanations: differences in exposure levels, timing of exposures, or frequency may be responsible. Another important difference was that Stodolnik-Baranska [1967, 1974] used pulse-modulated microwaves, whereas Roberts et al. [1983] used continuous-wave exposures. This last difference seemed to be a singular one, as in at least two other instances [Czerski et al., 1974; Kues et al., 1985] enhancement of biological effects was reported following exposures at the same frequency and same average dose rate, the only difference being modulation: pulsed vs. continuous waves, whereas in a third study in which microwave-induced temperature elevation was prevented [Stewart-DeHaan et al., 1983] pulsed microwaves induced effects comparable to those induced by large conventional temperature elevation.

Czerski et al. [1974] found that CW or pulsed, 2,950-MHz (1-μs., 120-pps, 2-h daily) exposure of rabbits induced changes in iron transport and turnover rates as well as iron incorporation into erythrocytes. PW exposure for a total of only 74 h induced changes comparable to those induced by an exposure to CW 2950 MHz at the same average power as the PW exposure but continued for more than twice the duration (158 h). Kues et al. [1985] examined ocular effects in Cynomolgus monkey of 2450-MHz microwaves, either pulsed or continuous wave. Pulsed microwaves at an average power density of 10 mW/cm^2 (SAR = 2.6 W/kg) produced the same effects as CW exposure at 20–30 mW/cm^2 (SAR = 5.3–7.8 W/kg). Stewart-DeHaan et al. [1983] examined rat lenses exposed to 915-MHz pulsed microwaves and compared the effects to the thermal injury obtained by conventional heating. They found changes in lenses exposed to microwaves at 37°C, i.e., at the level at which no thermal injury occurs. In lenses exposed at 39°C the extent of changes observed in the lenses was equivalent to that induced by conventional heating to 47° or 50°C.

These observations indicate that pulse modulation enhances effects on biological structure and function beyond what is observed with CW microwave exposure or conventional heating. In the present study spontaneous lymphocyte transformation obtained by pulsed or CW microwaves and conventional heating has been compared, and its dependence on the exposure conditions investigated.

MATERIALS AND METHODS

The exposure system and dosimetry have been described in detail elsewhere [Joyner et al., 1989]. The exposure system consisted of a shorted section of single-mode S-band waveguide (WR430, internal dimensions 109.22 mm × 54.61 mm), in which the samples were placed, fed from a modulatable microwave source. A second

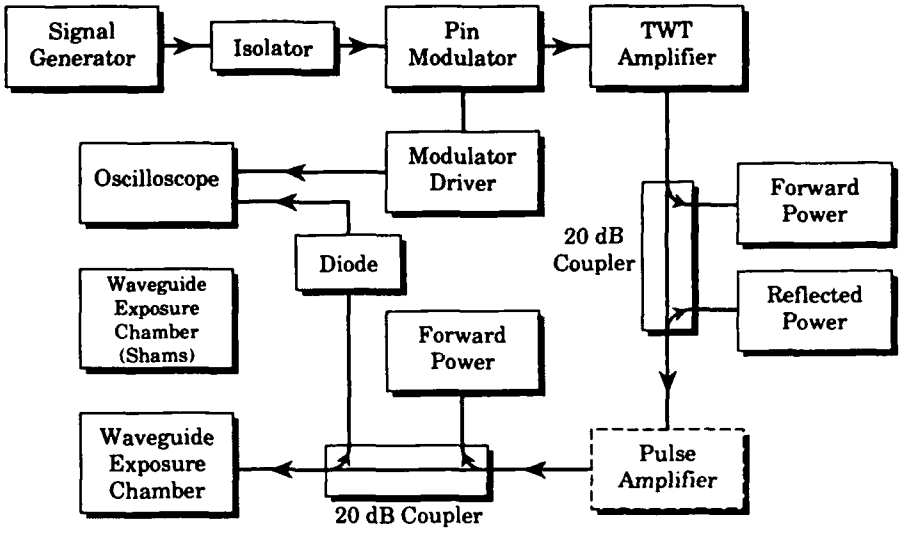

Fig. 1. Schematic diagram of essential components of microwave exposure system.

unexcited waveguide, identical to the first, served to house samples used as controls. At all times controls were studied simultaneously with exposed samples. Exposure parameters such as input microwave power and sample temperature were recorded for automated, on-line dosimetry.

The waveguides were placed in a conventional CO_2 tissue culture incubator (Forma Scientific). One waveguide was used for sham exposure, the other one was connected to the microwave power source. A schematic diagram of the exposure system with its monitoring elements is given in Figure 1. Control and monitoring of exposure powers and waveforms perform only an auxiliary function, however, as the conditions of exposure are characterized by dosimetry based on measurements of temperature and of SAR within the sample.

Pulsed exposures involved 1-μs-duration square-envelope bursts of microwaves at 100–1,000 pulses per second. The pulse length was chosen as it was the shortest we could conveniently generate; the pulse repetition frequency was selected to provide easy adjustment of the SAR over the range required. We plan in a future study to explore further the relationship between lymphoblastoid transformation and the pulse characteristics in a PW exposure.

The dosimetric part of the system consists of two minimally perturbing temperature probes, connected to a Hewlett-Packard Model-59306A relay activator, which is itself connected to a Keithley Model-192 digital voltmeter (DVM). Both the relay activator and the DVM are under the control of a Hewlett-Packard Model 86 desk-top computer through the HPIB (IEEE-488) bus. Initially a Narda Model-8011B non-perturbing double temperature probe was used; later, two Vitek Model-101 probes were substituted for the Narda probe.

The temperatures of exposed and sham-exposed samples were recorded sequentially, under computer control, with a minimum interval of 1 s, and stored. The temperature was recorded before the beginning of, during, and after the exposure. The "on" and "off" times of exposure were recorded on the computer by the operator using a "soft-key" interrupt capability. Because the temperature of the

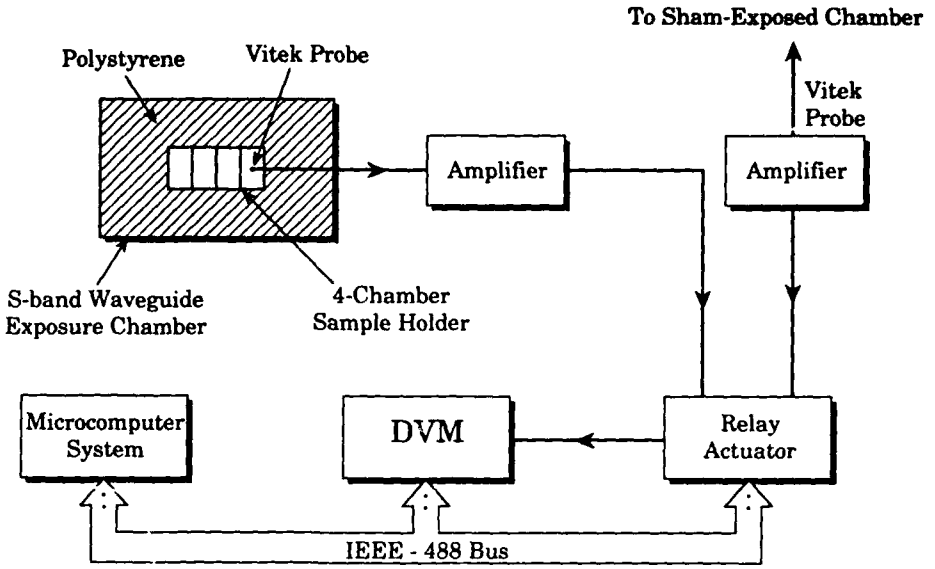

Fig. 2. Schematic diagram of arrangement for sample temperature monitoring and dosimetry.

sham-exposed sample was essentially constant, in view of the stability of the temperature in the incubator, it was monitored less frequently than the temperature of the exposed samples. At the conclusion of a run the $T(t)$, i.e., temperature (T) versus time (t) behavior was analyzed to determine the SAR. This measure was computed automatically from analysis of the heating-cooling curves, via software developed in-house for this purpose. The oscillations of the temperature over 24 h did not exceed ± 0.2 °C. For conventional thermal exposures, the temperature could be elevated by increasing the temperature of the incubator, maintaining control samples in another CO_2 incubator.

A four-chamber slide (Lab-Tek, Miles Laboratories, Model No 4804) served as a sample holder. This small sample holder occupies only 16% of the cross-sectional area of the waveguide, and in theory should provide SAR uniformity of ± 12.5%. Before any actual cell exposures were made, careful measurements of the SAR in each of the four sample holder wells were made. These measurements demonstrated that the differences in energy deposition between particular chambers remained within ± 10%. Three of the chambers contained cells and media; the fourth (end chamber) contained medium only. A hole was drilled in the cover of this chamber and the temperature probe was introduced. Therefore, each exposure series was carried out in triplicate on simultaneously exposed samples with simultaneous continuous dosimetry. Observations at a given SAR with CW or PW exposure involved several such exposure series, the actual number varied depending on the reproducibility of the measured SARs.

Figure 2 is a block diagram of the dosimetric system and Figure 3 shows a waveguide with the sample holder inserted. Note that the sample holder is symmetrically placed in the center of the waveguide and is oriented so that the electric field of the TE_{10} mode in the waveguide is parallel to the long dimension of the sample holder wells.

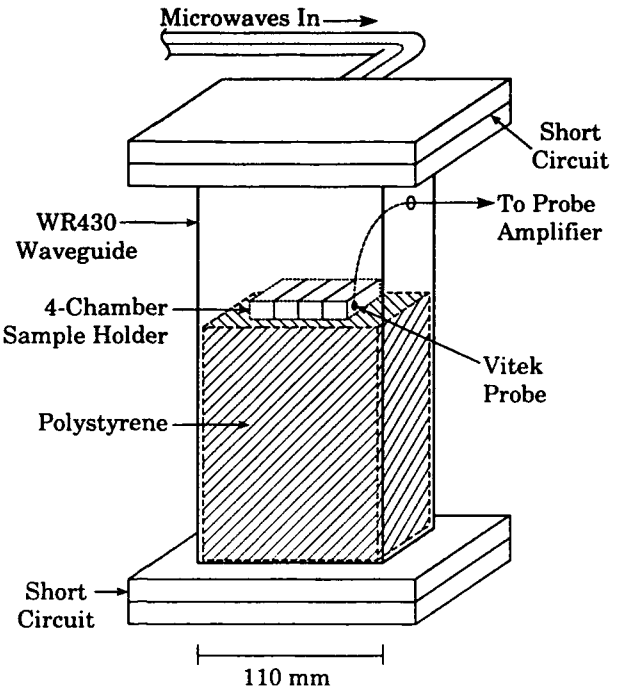

Fig. 3. Waveguide exposure chamber showing placement of four-chamber sample holder and temperature probe.

Lymphocyte Cultures

Approval for the use of human blood was obtained from the Human Subjects Research Review Board (HSRRB), Office of the Surgeon General, Department of the Army. Volunteers were informed about the nature of the study, potential risks, and the potential benefit derived from the study, and they signed the informed consent form as approved by the HSRRB.

Twenty ml of blood was withdrawn by venipuncture under aseptic conditions into two heparinized (143 USP units) sterile evacuated blood collection tubes (Vacutainer No. 6480, Becton and Dickinson). The blood was transported to the laboratory at room temperature. Within 1 hour after withdrawal, mononuclear cells (lymphocytes and monocytes) were separated under sterile conditions from the whole blood by gradient centrifugation in Lymphocyte Separation Medium (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. After this procedure there were no detectable red blood cells present. Following separation the cell suspension was washed twice in RPMI 1640 cell-culture medium (Gibco). An aliquot was withdrawn for the determination of the cell concentration, cell viability, and cytological (cytocentrifuge) preparations; the remainder was used for establishing cultures.

The cells were counted in a bright-line hemocytometer (Neubauer type, Spencer). Viability was tested by the trypan-blue exclusion test, neutral red stain for the vacuome, and Janus green stain for mitochondria. Cytocentrifuge (Cytospin2, Shandon) preparations were made, air dried, fixed in methanol, stained by a combined

Wright-Giemsa stain, and cell morphology was analyzed under a microscope. Four-chamber culture-chamber slides were filled under sterile conditions with 1 ml of chromosome medium 1A (Gibco) without PHA. One million cells each were added to three of the chambers, the fourth held medium only. Following incubation the cultures were mixed within the culture chamber using a Pasteur pipette and transferred to microfuge tubes. The cell count was established, viability was tested, and cytocentrifuge preparations were made.

There was no difference in viability between cells incubated in the exposure system and cells from the same donor that were cultured in a conventional manner. A triplicate control and exposure series on a given day would use lymphocytes obtained from the same donor. However, during the course of the experiments blood from several donors was used.

For chromosomal preparations the cultures were harvested after 72 h of incubation with colcemide (Gibco) added 3 h before harvest. Cells were fixed, and placed on microscope slides for staining and examination.

ANALYSIS AND EXPERIMENTAL DESIGN

Analysis of Lymphoblastoid Transformation

Lymphoblastoid transformation has been evaluated on the basis of morphological features of cells [Ling and Kay, 1976] and by incorporation of tritiated thymidine [Hume and Wiedeman, 1980; Knight, 1987]. The use of tritiated thymidine only allows data to be obtained concerning the number of cells in the S phase and their proliferative potential [Cleaver, 1967]. Morphological traits allow one to evaluate the course of transformation over successive stages, thus it is not only as good a criterion as thymidine incorporation, but provides a fuller scope for evaluation including subtle changes. Therefore, morphological features were selected for the evaluation of lymphoblastoid transformation. The principal drawback of this approach is that the evaluation of cell morphology is very subjective. To eliminate this and to allow quantitation and statistical analysis, image analysis was used.

The transformation process involves gradual enlargement of the cell and of the nucleus. At the same time the dense chromatin structure of the small lymphocyte becomes less dense and the cell passes into the intermediate stage. Nucleoli become recognizable within the nucleus among the skeins of chromatin. Finally, the lymphoblastoid form becomes a very large cell with a large nucleus containing several distinct nucleoli. Lymphoblastoids divide into two daughter cells with morphological features of small lymphocytes. The whole process is a cellular response to autigenic or lectin stimulation, and shows dose dependence [Ling and Kay, 1976; Hume and Wiedeman, 1980].

The morphological features of the cells were analysed in air-dried Wright-Giemsa stained cytocentrifuge preparations. The use of the cytocentrifuge ensured that the cells were evenly spread, their morphology uniform, and comparable between individual slides. A Zeiss Photomicroscope III equipped with an 100× oil immersion objective and 10× ocular was used. An image analysis system (Optomax) was mounted on the microscope. Three different observers were assigned coded preparations from cultures incubated at different PHA concentrations. The observers scored the preparations blindly, measuring the total cell area, total nucleus area, computing the nucleo-cytoplasmic ratio, and assigning a symbol characterizing the morpholog-

TABLE 1. Distribution of Cell Sizes in Cultures Incubated With Different PHA Concentrations

	PHA μg/ml		
Area (a) μm^2	5.0	7.5	10.0
$a \leq 100$	42.5 ± 3.7	26.8 ± 4.5	17.4 ± 2.5
$100 < a \leq 120$	13.4 ± 2.0	13.0 ± 2.8	5.3 ± 1.1
$120 < a \leq 150$	15.6 ± 3.4	12.1 ± 3.1	10.0 ± 2.1
$150 < a \leq 200$	19.0 ± 2.6	24.3 ± 2.8	22.6 ± 3.0
$a > 200$	10.6 ± 4.8	25 ± 3.5	46.2 ± 4.5

ical appearance of the cell (S-small, I-intermediate, B-blastic form). The results obtained in the series described below were examined and compared using the Statgraph software of Statistical Graphics Corporation.

In a series of preliminary experiments PHA was added at a concentration of 5.0, 7.5, or 10.0 μg/ml, of PHA in medium "chromosome 1A" and the preparation analysed as described above; 10 μg/ml is the optimum concentration determined by the manufacturer for lymphocyte transformation. Based on this analysis, the total cell area was selected as the most informative measure for the evaluation of transformation. Other measurements did not provide additional information or were only partially informative. Taking all the information into account some arbitrary decisions were to be made. Lymphocytes with a total area below 100 μm^2 were assigned to the category of small lymphocytes, cells of the area greater than 100 and smaller or equal to 200 were considered intermediate forms and subdivided further into three subclasses (see Table 1). Cells with a total area greater than 200 μm were assigned to the category of lymphoblastoids. Table 1 presents the results of this series of experiments.

Statistical analysis was carried out as recommended by Knight [1987; see also Ling and Kay, 1976; Hume and Wiedeman, 1980]. Results were analyzed using Statgraph software. Analysis of Variance (ANOVA) was used to compare the number of cells in particular classes. Distributions were compared using multiple regression methods. Results were judged significant at the $P < .05$ level.

Table 1 demonstrates a statistically significant ($P < .02$) decrease in the numbers of cells with an area below 100 μm^2 with increasing PHA concentration. No differences were seen between the numbers of cells with an area between 100 and 200 μm^2, except for the cells with an area between 100 and 120 μm^2, following incubation with 10 μg/ml PHA. The number of such cells under these conditions decreases by a factor of 2.

Cells with an area above 200 μm^2 increased with increasing PHA concentration, presenting a "mirror image" of the effect on the number of small lymphocytes. In brief, the shifts in the numbers of lymphoblastoid cells occurred at the expense of small lymphocytes. These results validate the morphological approach selected for the evaluation of lymphoblastoid transformation. It should be noted that Michaelson et al. [1982] came to the same conclusion, and maintained that tritiated thymidine incorporation and analysis of cell morphology may be used alternatively for quantitation of lymphoblastoid transformation.

Experimental Design

Each experiment involved three chamber slides: one exposed, a second one sham-exposed, and a third one incubated in a separate incubator at 37°C. Thus in

each experiment, there was one experimental sample and two control ones. Each sample holder (chamber slide) had three chambers containing 1 ml medium with 10^6 cells suspended in each and one chamber containing medium only. This chamber served for the introduction of the temperature probe. With this design each basic condition, experimental or control, was performed in triplicate. At a particular CW or PW exposure level, several experiments were performed, at least two, to check for consistency in the results of morphological examination. Results were pooled following analysis. In each instance cells were harvested for cytocentrifuge preparations following a 5-day period of incubation. Using the above protocol, we carried out the experiments in three series:

Series 1. Cultures exposed to CW 2450 MHz at non-heating and heating levels of exposure. The nonheating level corresponded to an average SAR of about 1 W/kg, ranging from 0.8 to 1.3 W/kg when the variations between experiments or between individual chambers were taken into account. The heating levels resulted in an increase in the temperature of the sample by about 0.5°C (average SAR 1.8–2.3 W/kg), by about 1°C (SAR 3.5–4.5 W/kg), by about 1.5°C (SAR 6.8–8.3 W/kg), and by about 2°C (SAR 9.8–12.3 W/kg). The temperature of the sample varied over the incubation period by ± 0.2°C. In a few instances the variations reached ± 0.5°C.

Series 2. Samples exposed to PW 2450 MHz at non-heating levels and heating levels that resulted in the same temperature increases, and consequently in the same average SARs, as in series 1. The pulse width was 1 μs. The pulse repetition rate varied from 100 to 1,000 pps as the exposure conditions changed from the non-heating to heating levels. At the maximum average SAR, at 1,000 pps, the peak SAR was from 9.8 to 12.3 kW/kg, which corresponds to a deposited energy of 9.8–12.3 mJ/kg per pulse. This quantity of energy was sufficient to raise the sample temperature by only $2.3–3.2 \times 10^{-6}$°C after a single 1-μs pulse. The thermalization time constant of the samples, which can be calculated from their size and thermal diffusivity, was ~0.5 s. Consequently, there was no significant impulsive heating of the samples. However, in view of the rate of temperature change during each pulse, which is $2.3–3.2 \times 10^{-6}$°C, there remains a possibility of the generation of an acoustic disturbance in the samples. This would be a manifestation of the photoacoustic effect in which the absorbed radiation is in the microwave region. These phenomena have been known since the last century [Rosencwaig, 1980] and were first reported in the microwave region in 1963 [White, 1963]. However, the expected acoustic disturbances are very weak and it remains problematical to explain the phenomena we observed in terms of their effect.

Series 3. Samples heated by conventional means to obtain temperature increases as in series 1. Samples incubated in a separate incubator at 37°C served as controls.

It is worth pointing out that the maximum SAR variation from chamber to chamber in the microwave exposures, ± 12.5%, can account for only a 0.4°C temperature difference between the inner and outer chambers of the four-chamber sample holder at the highest SAR used.

RESULTS

No significant differences ($P < .8$) were observed between sham-exposed and control cultures. Consequently, the results obtained from these cultures were pooled

TABLE 2. Distribution of Cell Sizes in Control and Sham-Exposed Cultures Incubated for 120 h

Area(a) μm^2	Number of cells mean ± s.d.
$a \leq 100$	53.7 ± 2.9
$100 < a \leq 120$	30.9 ± 1.8
$120 < a \leq 150$	10.1 ± 1.0
$150 < a \leq 200$	2.1 ± 0.8
$a > 200$	1.2 ± 0.9

and are presented in Table 2. In this and subsequent tables, the number of cells in each classification based on cell area represents the number of cells per 100 counted cells that belong to each classification. In these tables the number of cells of each classification in each group does not add up to exactly 100 because these numbers represent a statistical analysis of numbers of triplicate exposures, plus there are always a few cells that are difficult to classify. These results served as basic reference control data for the evaluation of lymphoblastoid transformation. It should be noted that lymphocytes undergo spontaneous transformation at a very low level: 53.7 ± 2.9 cells is the mean number per 100 for small lymphocytes. The numbers of intermediate forms decrease gradually with increasing cell area. Lymphoblastoids occurred in 1.2 ± 0.9 per 100.

Table 3 presents the distribution of cell sizes in conventionally heated cultures. No differences were observed between control and sham-exposed cultures (37°C) and cultures elevated in temperature by a further 0.5°C ($P < .3$). At 38°C the number of small lymphocytes decreased and the number of cells with areas between 100 and 150 μm^2 increased ($P < .05$), thus indicating an enhancement of the transformation process. At 38.5°C intermediate forms with areas between 100–120 μm^2 decreased and the number of cells with an area between 150 and 200 μm^2 increased ($P < .001$), but no blastoid cells were found. This may be interpreted as an enhancement of transformation or as an inhibition. The latter interpretation is less likely because of the results obtained at 38°C. No signs of cell destruction were observed in this series; however, it is well known that conventional heating above the levels that we used would lead to cell destruction. At 39°C there was a significant increase ($P < .002$) in the number of small lymphocytes, but not in the number of blastoid cells.

Table 4 presents the distribution of sizes in cultures exposed to CW 2450-MHz microwaves at heating and non-heating levels. A significant decrease ($P < .005$) was observed in intermediate forms with areas between 100 and 120 μm^2 at 38.5°C. This decrease was accompanied by an increase ($P < .02$) in forms with an area between 120 and 150 μm^2 at 38°C, and a highly significant increase ($P < .001$), by a factor of between 4 and 10, in forms with an area between 150 and 200 μm^2 at 38.5°C. These shifts indicate enhancement of blastic transformation as expressed by shifts in intermediate forms.

At 39°C the interpretation of preparations becomes unreliable, as partial cell destruction occurs. Lymphocyte shadows may survive in a very capricious manner and our previous experience indicates that viability tests have large margins of errors. Therefore no analyses were carried out at 39°C. It is possible, but not certain, that at

TABLE 3. Distribution of Cell Sizes in Control Cultures Incubated at 37, 37.5, 38, 38.5, and 39°C With Conventional Heating

	Number of cells mean ± s.d.				
	Incubation temperature				
Area (a) μm^2	37°C	37.5°C	38°C	38.5°C	39°C
$a \leq 100$	55.2 ± 3.8	54.8 ± 2.2	39.6 ± 3.0	53.0 ± 4.5	88.7 ± 2.2
$100 < a \leq 120$	32.4 ± 2.9	29.4 ± 3.6	29.6 ± 7.1	14.7 ± 2.3	9.1 ± 1.9
$120 < a \leq 150$	8.8 ± 2.0	11.2 ± 1.7	23.2 ± 3.4	16.2 ± 2.2	1.6 ± 1.4
$150 < a \leq 200$	1.6 ± 0.9	1.3 ± 1.0	3.4 ± 1.0	15.8 ± 2.0	0.3 ± 0.5
$a > 200$	1.0 ± 0.9	2.4 ± 1.7	4.4 ± 1.0	0	0.1 ± 0.3

TABLE 4. Distribution of Cell Sizes in Cultures Exposed to CW 2450 MHz Microwaves at Nonheating (37°C) and Heating Levels

	Number of cells mean ± s.d.				
	Incubation temperature				
Area (a) μm^2	37°C	37.5°C	38°C	38.5°C	39°C
$a \leq 100$	53.3 ± 2.1	40.0 ± 3.4	39.4 ± 3.0	43.6 ± 4.3	Partial cell
$100 < a \leq 120$	31.7 ± 3.0	36.0 ± 1.9	30.0 ± 4.9	15.2 ± 2.7	destruction
$120 < a \leq 150$	10.2 ± 2.4	12.2 ± 2.0	22.8 ± 2.8	17.6 ± 1.7	measurements
$150 < a \leq 200$	1.7 ± 0.7	4.8 ± 1.6	2.1 ± 0.9	22.6 ± 2.8	unreliable
$a > 200$	1.0 ± 0.9	2.4 ± 1.7	5.7 ± 1.7	1.0 ± 0.9	

this maximum SAR level, the ~0.4°C temperature elevation of the inner sample chambers above the outer chambers could lead to partial cell destruction.

Comparison of Tables 3 and 4 demonstrates that there are no significant differences ($P > .05$) between the results obtained by conventional and CW 2450-MHz heating provided the temperature elevation is below 1.5°C, but at greater temperature elevations transformation is enhanced and partial cell destruction occurs. There is a slightly significant ($P < .1$) reduction in the number of small lymphocytes at 38.5°C when conventional and CW microwave heating are compared. The lack of significant differences reinforces the reliability of our dosimetry; if there were any significant temperature differences between the four measurement chambers, this would show up in the results. It is clear that temperature plays a significant role in changing the cell-size distribution.

Table 5 presents the results obtained in cultures exposed to PW 2450 MHz at heating and non-heating levels. There are no significant ($P > .1$) shifts in the numbers of small lymphocytes and intermediate forms with an area between 100 and 150 μm^2 in cultures at 37°C and those heated to 38°C. There is a gradual increase correlating well with heating in the number of cells between 150, 200, and 250 μm^2. The number of lymphoblastoid cells increased by a factor of 2. It should be noted, however, that at 37°C, the number of lymphoblastoid forms is significantly greater ($P < .02$ at 37°C, $P < .002$ at 37.5°C) than in cultures heated conventionally and by CW microwaves. This finding seems to indicate a much higher efficiency of enhancement of transformation by PW microwaves than by CW and/or conventional heating.

Partial cell destruction and lack of reliability in cell preparation evaluation occur at a lower level of heating then is the case for CW or conventional heating. Such effects become apparent at temperatures higher than 38°C and become pronounced at

TABLE 5. Distribution of Cell Sizes in Cultures Exposed to PW 2450 MHz Microwaves at Nonheating (37°C) and Heating Levels

	Number of cells mean ± s.d.				
	Incubation temperature				
Area (a) μm^2	37°C	37.5°C	38°C	38.5°C	39°C
$a \leq 100$	59.7 ± 3.9	44.6 ± 3.8	46.8 ± 4.7	Partial cell	
$100 < a \leq 120$	15.4 ± 1.8	20.6 ± 2.4	16.3 ± 2.0	destruction	Not
$120 < a \leq 150$	13.7 ± 1.4	16.6 ± 1.5	16.3 ± 2.0	measurements	performed
$150 < a \leq 200$	4.9 ± 1.8	3.9 ± 1.0	10.6 ± 2.0	unreliable	
$a > 200$	6.3 ± 1.7	14.4 ± 1.2	15.9 ± 3.7		

38.5°C. In view of this, experiments at higher temperature levels were not performed. This cannot be explained by small temperature uncertainties in the dosimetry as the temperature elevations and their spatial distribution are identical between CW and PW exposures.

There are only a few studies of the effects of heating on the immune responses of human lymphocytes in vitro. They were conducted mainly by the Rochester Group [Roberts, 1983; Roberts et al., 1983, 1984]. These investigations can be summarized with the statement that the effects of heating on the response to lectins or antigens consists of enhancement that starts to appear within the range of a temperature increase of 0.2–0.5°C. The results presented here are consistent with these findings with the difference that effects become detectable and statistically significant in our work when the increase in temperature of the medium exceeds 0.5–1.0°C. With temperature increases above 1°C and PW modulation, cell destruction occurs and is highly likely to interfere with cell function in vitro.

Studies on the effects of heating, be it conventional or be it by microwaves, are comparable between the above sets of experiments within experimental limits. Studies on the effects of microwave exposure conducted by the Rochester Group and by Cleary et al. [1990] can not be readily compared. The experiment conducted by the Rochester Group consisted in exposure of human lymphocytes for 2 h before stimulation with PHA, subsequent addition of PHA and evaluation of the preparations extended by determination of thymidine incorporation. Thus the examined effect of exposure concerns the interaction of cells with electromagnetic fields during the G0 phase. Such an interaction would have to lead to long-term after effects to become detectable during the S phase. The experiments of Cleary and coworkers were performed at substantially higher SAR levels with simultaneous cooling of the preparations. With this protocol the after effects of exposure during the G0 phase were observed. This may be related to the difference in the SARs used by Cleary and the Rochester group.

The mechanisms involved in the reported phenomena are unknown and difficult to interpret. The primary site of interaction may reside in the cell or nuclear membrane, or even at the level of genetic information. Thus only limited conclusions may be drawn from this work. The mechanism by which pulsed exposures lead to different biological effects is not certain. Pulsed exposures do not lead either to macroscopic or to microscopic heating that is significantly different from CW exposure at the same average power level. However, our pulsed exposures do produce much larger rates of change of temperature, albeit for a very short time. Whether such rapid temperature

changes lead to micro-acoustic shock effects, or whether the effects are caused directly by the much larger field amplitudes in the pulsed exposures cannot be determined from our observations.

CONCLUSIONS

1. CW 2450-MHz exposure at physiological temperatures (~37°C) at nonheating levels does not affect spontaneous lymphoblastoid transformation.

2. Conventional and CW heating enhance lymphoblastoid transformation to the same degree under small temperature elevations. However, CW microwave heating leads to cell destruction at apparently smaller temperature elevations than is the case for conventional heating, which might result from small temperature differences between these two exposure modalities.

3. PW exposures that lead to the same increase in temperature and energy absorption as CW exposure performed in a preceding series enhance lymphoblastoid transformation to a significant extent.

4. The most notable finding is enhancement of lymphoblastoid transformation at a nonheating level caused by PW exposure, whereas no such enhancement was observed following CW exposure.

5. The above conclusions can be summarized in the statement that PW and CW 2,450-MHz exposures act differently in causing spontaneous transformation of human lymphocytes in vitro.

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EFFECTS OF ELF ON C-MYC ONCOGENE EXPRESSION IN NORMAL AND TRANSFORMED HUMAN CELLS

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Abstract: Cell cultures of the human lymphoma cell line, Daudi, were exposed to 2.5, 5, 7.5,or 10 Gauss extremely low frequency magnetic fields. Transcription of the genetic marker, c-myc, was determined in cultures exposed for various time periods. Increases of c-myc transcripts were detected in some exposed cell cultures. The increase of the transcripts appeared to show dependence both on the applied field and exposure duration.

INTRODUCTION

Epidemiological, animal, and in vitro studies suggest linkage between exposure to 60 Hz sinusoidal electromagnetic fields (EMFs) and incidence of cancer (see review in [4]). The main concern is exposure to transmission power lines and to several electric devices. The existing data are still controversial, as they do not fully support the idea of extremely low frequency (ELF) EMFs being a cancer promotor. The data also do not exclude this possibility. In addition, no mechanistic basis has been established for ELF as a cancer promoting agent; this leads to further controversy.

Exposure to ELF EMFs is known to increase the level of several gene transcripts. Reba Goodman and coworkers [2,5] showed changes in gene transcription in Drosophila salivary gland cells and in HL-60, a human promyelogenous leukemia cell line (beta-actin, histone 2B and c-myc). Jerry Phillips [3] reported increased transcription of the genes encoding c-myc, c-fos, c-jun, and protein kinase C in human cell lines. The utilization of various exposure systems and the characteristics of applied fields, as well as differences among biological models, lead to problems in comparing results. However, the above papers report increased gene transcription following exposure to ELF EMFs.

We focused our research on transcription of the c-myc oncogene. We have examined transcription in two transformed human cell lines (Daudi lymphoma and HL-60) and in normal human peripheral lymphocytes. Transcription was examined by Northern blot hybridization analysis. Exposure for 30, 45, 60, or 180 min. to 60 Hz continous waves at 1 gauss resulted in increased expression of c-myc oncogene expression in Daudi cells. No appreciable changes were noticed in c-myc expression in HL-60 and peripheral human lymphocytes. The presence of a chromosomal translocation involving the c-myc locus in the Daudi cells [6] be an explanation for the increased c-myc transcription in this cell line.

We have expanded our experiments on Daudi cells by introducing different exposure parameters. In the present experiments, cells were exposed to 2.5, 5, 7.5, or 10 gauss, 60Hz sinusoidal EMFs. Positive results were obtained.

MATERIALS AND METHODS

Human cancer cell lines were obtained from the American Type Culture Collection, Rockville, MD 20852, and cultured in RPMI-1640 medium with 20% fetal bovine serum. Cells were grown in an CO_2 incubator, 5% CO_2 to a density of $5x10^5$/ml, and resuspended at the same cell density in fresh medium 1 hour prior to ELF exposure. Cell suspensions were placed in 30 ml aliquots in Corning T75 tissue culture flasks and exposed for 15, 30, or 60 min. to 2.5, 5, 7.5, or 10 gauss (G) 60 Hz sinusoidal EMF in the incubator, 5% CO_2, at 37° C. Each exposure flask was accompanied by an identical control flask that was placed in the μ metal box in the same incubator. After exposure, total RNA was immediately extracted with guanidinium isothiocyanate and the RNA pelleted by CsCl step gradient ultracentrifugation. The purified total RNA samples were then run in a denaturing formaldehyde agarose gels and transferred to polymer membranes (Northern blot separation technique). Phosphorus-32-labelled DNA c-myc and beta-actin probes were used to hybridize against the Northern blots. The commercially available c-myc probe (Oncor, Inc.) showed specificity with RNA in our laboratory. However, commercial beta-actin (Oncor, Inc.) was not adequately specific, so we used a probe prepared at the Medical College of Virginia, Richmond. The RNA samples from both exposed and control cells were probed at the same time. Autoradiography was carried out at -70° C for 2-3 days. A laser scanning densitometer was used to quantitate the intensity of hybridization of the two probes. The ratio of densitometer values, exposed : control, was determined. Data are presented as that ratio. Values were normalized to densitometer values of beta-actin.

RESULTS

Analysis of Northern blots showed increased expression of c-myc oncogene following 60 min. exposures to ELF. The increase is apparently dose dependent, with the exposed : control ratios varying from 1.62 at 2.5 G and 1.75 at 7.5 G, to 2.67 at 10 G exposures. Consistent patterns of reactions were not observed after exposures at other applied fields and for other exposure durations. The results are presented in Table 1.

Table 1. Laser densitometric quantitation of c-myc expression normalized to beta-actin to compensate for difference in sample loading. Ratio exposed : control.

Exposure Time (min)	Applied Fields, G		
	2.5	7.5	10
15	1.16	0.90	1.29
30	1.31	1.01	1.42
60	1.62	1.75	2.68

Long exposure durations, i.e., 3, 6, or 12 hours, did not show any differences between exposed and control samples. This was an expected result, inasmuch as the gene transcripts are short-lived.

DISCUSSION

Previously published data on the exposure of normal and transformed human cell lines [1] showed increased c-myc expression following 1 hour exposure to 60 Hz sinusoidal EMF in Daudi lymphoma cells known to carry a translocation in c-myc locus on the chromosome. No differences in c-myc expression were observed in the HL-60 cells and in human peripheral lymphocytes. The expression of c-myc appeared to depend on the dose and on duration of the applied field. Although Northern blot analysis provided relative data about specific gene products, quantification is difficult. Some sources of variation include variations in dosimetry readings for exposure of cultures, in autoradiography, and in densitometry. Other methods should be employed to measure specific levels of transcripts, in order to reduce variability and to provide a clear basis for interpretation.

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COMPARISON OF THE EFFECTS OF EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELD EXPOSURE TO INTACT HUMAN CELLS OR THEIR NUCLEI WITHOUT CYTOPLASMIC MEMBRANE

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ABSTRACT

Using flow cytometry with acridine orange, the effects of ELF exposure on RNA fluorescence was assessed. Intact Daudi cells respond to ELF field exposure by increasing total cellular RNA fluorescence, but, their isolated nuclei did not. The Isolated nuclei of HL-60 cells also did not respond to ELF exposure as well as the intact HL-60 cells. The results obtained are consistent with the hypothesis that cytoplasmic membranes may play a role in mediating the transcriptional effect observed in Daudi cells when exposed to ELF fields.

INTRODUCTION

The cytoplasmic membrane has been proposed as the site of interaction between electromagnetic fields and biological systems (1). To rigorously test this hypothesis, we exposed intact human cells and their nuclei, stripped free of the cytoplasmic membrane, to 60 Hz 1 gauss fields and examined the transcriptional effects which occur in the nucleus of the cell.

MATERIALS AND METHODS

Daudi, a human lymphoma cell line harboring translocated c-myc oncogene, and HL-60, a human promyelogenous leukemia cell line containing normal c-myc proto-oncogene were used for this experiment. Nuclei were prepared according to a published procedure (2) with modifications. Cells were washed twice with RSB buffer (10 mM Tris-HCl, Ph 7.4, 10 mM NaCl, 3 mM $MgCl_2$). Two ml of RSB + 0.% NP40 + 2.75 mM DTT were mixed with each ml of cell suspension. The cells were then centrifuged briefly at 1,000 rpm for 1 min. The excess volume over 1 ml was discarded. 20 U of RNasin were then added and the mixture was homogenized by one or at most two strokes in a 1 ml homogenizer fitted with a B pestle. The mixture was then pipetted up and down through a Rainin blue pipet tip a few times. The nuclei were collected by centrifugation and suspended in RPMI-1640 + 20% fetal bovine serum (FBS).

One hour prior to exposure, approximately, 1 X 10^6 Cells or nuclei were placed in 20 ml fresh medium containing RPMI-1640 plus 20% FBS. After one hour pre-incubation, intact cells or nuclei were exposed to 60 Hz, 1 gauss for 1 hour in 5% CO_2 at 37°C. Immediately post-exposure, 50% ice-cold ethanol (v/v) was added to the cells or nuclei for fixation. Cells or nuclei were washed twice in PBS and 0.2 ml of the cell or nuclei suspension were then permeabilized with 0.4 ml of ice-cold solution containing 0.1% triton X-100, 0.05 N HCl and 0.15 N NaCl. The suspensions were mixed gently and then 1.2 ml of acridine orange (6 ug/ml) in phosphate citrate buffer were added to the suspension. After standing at room temperature for 5 minutes, the stained cells or nuclei were filtered through a 37 micron mesh and analyzed for RNA content using laser flow cytometry.

To confirm that nuclei are transcriptionally active and to assess ELF effects on specific gene transcripts in intact cells and isolated nuclei, nuclear run-off assays were performed by either exposure to intact cell for 60 min. and then isolate the nuclei and running the assay or by first isolating the nuclei, expose to ELF for 60 min. and then proceed to run the nuclear run-off assay. The labeled transcripts generated were hybridized against dot blots of various cloned DNA probes. Post hybridization and washing, the blots are autoradiographed for 14 days at -70°C. The labeled transcpits were also electrophoresed in a 8 to 25% denaturing polyacrylamide gel to ascertain the size range of transcripts generated by intact cells and isolated nuclei.

RESULTS

Daudi lymphoma cells responded to ELF exposure by exhibiting increased RNA fluorescence (Fig. 1) but their nuclei did not (Fig. 2). No response was observed in HL-60 intact cells (Fig. 3) or their isolated nuclei (Fig. 4) when exposed to ELF. This data is consistent and supportive of the hypothesis that cytoplasmic membrane plays a role in mediating ELF interaction

with the living cell. To further characterize the effect of ELF exposure on specific gene transcription with or without cytoplasmic membrane, we carried out nuclear run-off assays in intact cells and their corresponding nuclei using a new cell line, R3, which exhibit robust effect with ELF exposure. Exposure of that cell line to ELF exhibited an increased in the expression of three oncogenes – c-Ha-ras, N-ras and c-sis by 18.0,15.2 and 8.8 folds respectively (Table 1). Exposure of isolated nuclei of R3 cell line exhibited no effect on those three oncogene. Exposure of a non-responsive cell line, R4, exhibited no effect on those three oncogene transcripts (Table 2). Thus, this experiment further supports the hypothesis that cytoplasmic membranes play a role in mediating ELF interactions with that responsive cell.

DISCUSSIONS

It is interesting to note that we observed ELF fields activate three oncogene transcripts which are all associated with the cytoplasmic membrane and mediate signal transduction across the cell membrane. C-Ha-ras and N-ras code for G-proteins which are involved in signal transduction through the cytoplasmic membrane. C-sis oncogene codes for the beta chain of platelet derived growth factor. Thus, it is tempting to hypothesize that ELF exposure may induce a positive feedback loop to cause signal transduction pathways to amplify the ELF signal across the cytoplasmic membrane in an ELF responsive cell. This hypothesis may also account for the ELF responsive cell's ability to distinguish and amplify ELF signals with energy levels below kT.

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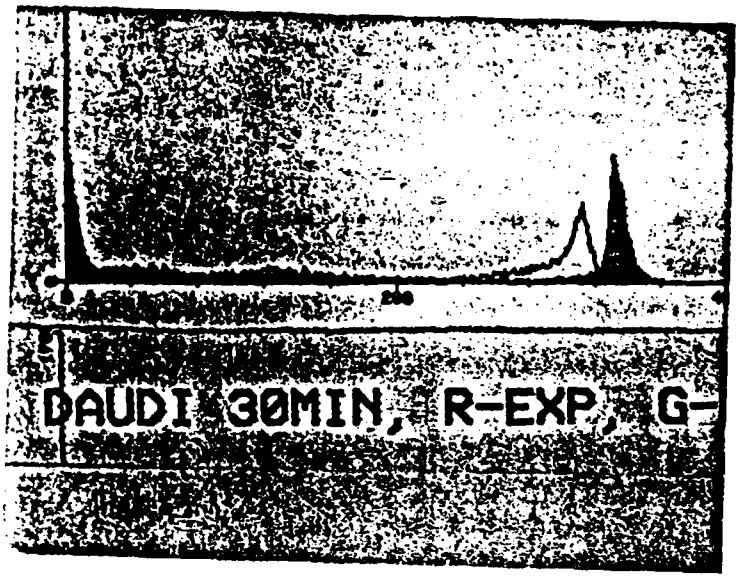


Fig. 1. RNA fluorescence histograms of intact Daudi cells (increasing RNA fluorescence is denoted by a shift to the right on ordinate)

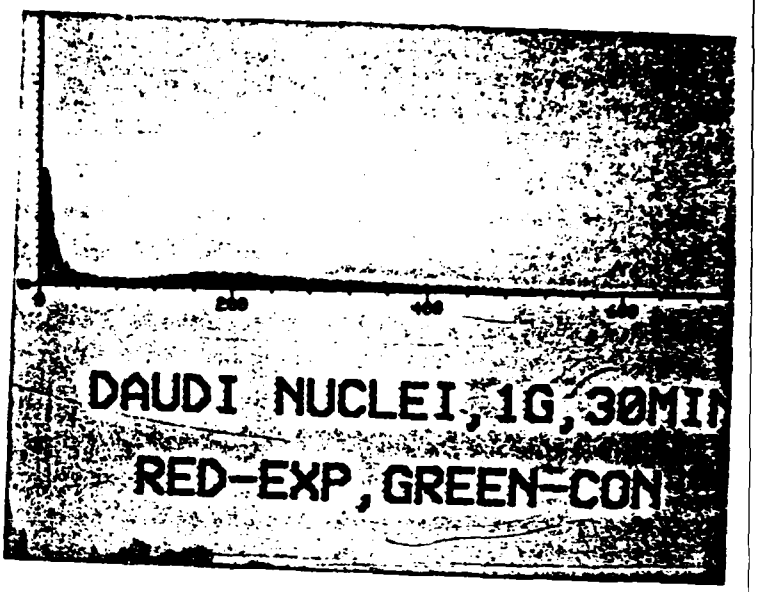


Fig. 2. RNA fluorescence histograms of Daudi nuclei.

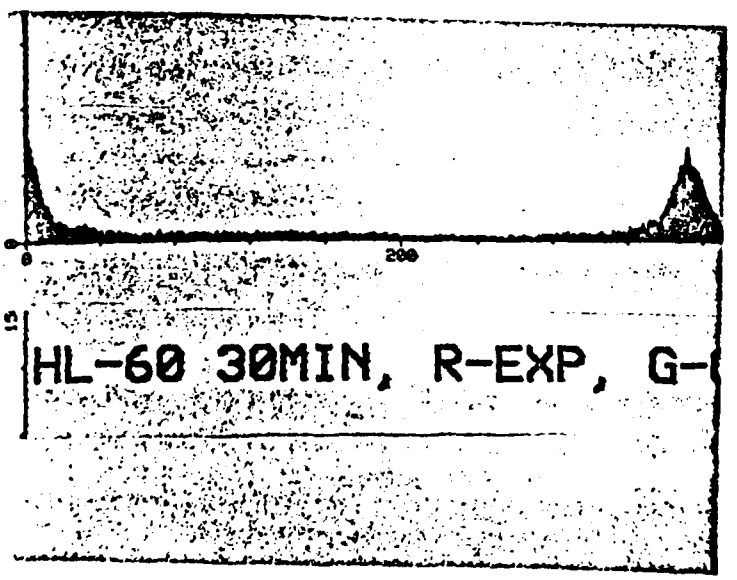


Fig. 3. RNA fluorescence histograms of intact HL-60 cells.

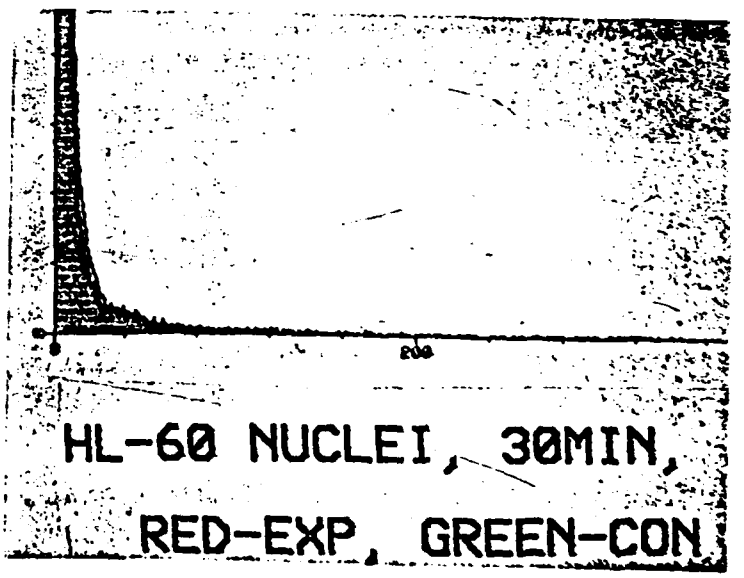
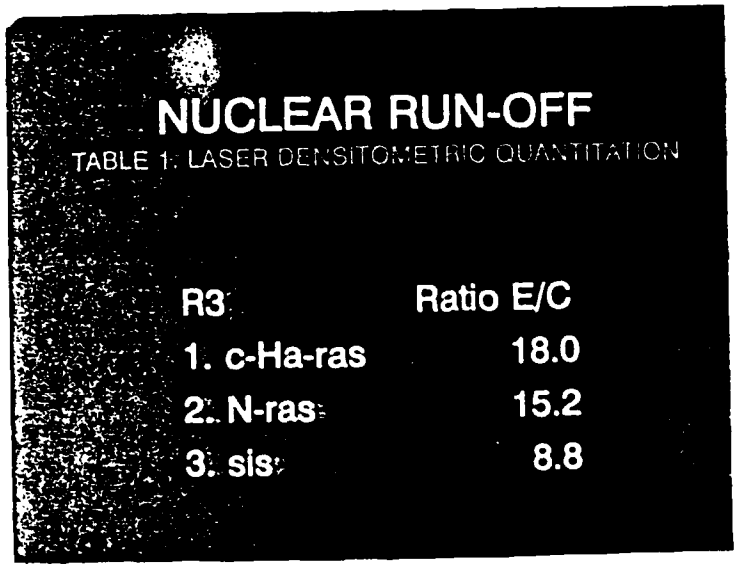


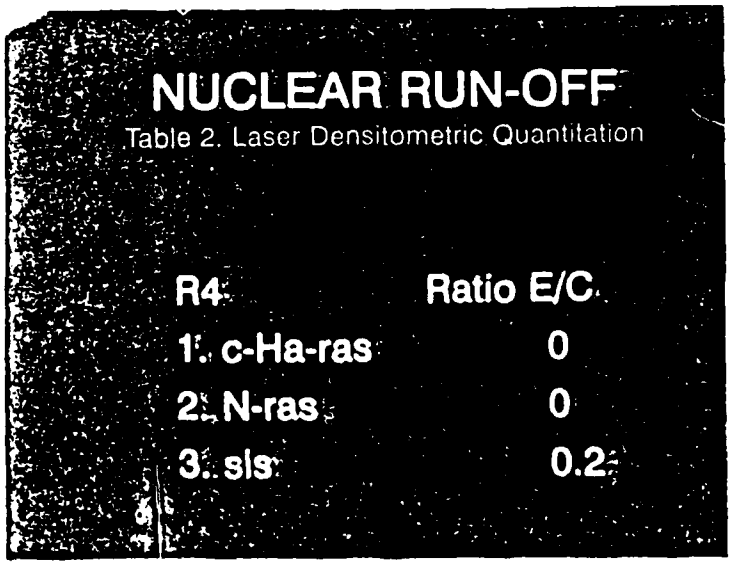
Fig. 4. RNA fluorescence histograms of HL-60 nuclei.

NUCLEAR RUN-OFF ASSAY

Labeled transcripts generated

	cpm/ul
R3, Exp	25230
R3, Con	22830
R4, Exp	13340
R4, Con	2340
R3 Nuc, Exp	34270
R3 Nuc, Con	22810
R4 Nuc, Exp	78540
R4 Nuc, Con	47250





THE EFFECTS OF VARYING THE FREQUENCY AND MAGNETIC FIELD STRENGTH ON MORPHOLOGIC PHENOTYPE OF ELF RESPONSIVE MODEL CELL LINE

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ABSTRACT

A model eukaryotic cell line with a marker gene under the control of a tentative ELF responsive DNA element was constructed in order to facilitate the investigations of various exposure parameters, e.g. dose–response, and frequency–response, that may be important in global characterization of the biological effects of ELF electromagnetic field interactions with living systems *in vitro*. Experiments using this model cell line which changes morphology as a result of exposure to ELF fields have yielded useful information on possible mechanisms of ELF interaction with living systems.

INTRODUCTION

A model eukaryotic cell line with a marker gene under the control of a tentative ELF responsive DNA element was constructed to facilitate the investigations of various exposure parameters such as dose–response and frequency–response which may be important in characterization and contribute to the understanding of the biological effects of ELF electromagnetic field interactions with living systems *in vitro*.

MATERIALS AND METHODS

The ELF responsive model cell line was constructed via transfection of primary adult rat Schwann cell isolated from sciatic nerve sheath and transfected with a plasmid containing SV–40 T antigen under the control of a metal regulatory element. An immortalized cell line was isolated. This cell line which is driven by the SV–40 T antigen oncogene expressed a round phenotype which is distinct from normal Schwann cell bipolar morphology with processes.

RESULTS

When this cell line is exposed to ELF in vitro, after 48 hours, the phenotype changes from the round cell shape (Fig. 1) with maximum cell density to bipolar morphology with the appearance of processes (fig. 2) and significant decrease in cell density. This response is observed when this cell line is exposed to 60 Hz at 1 gauss, a 15 Hz pulse trains and also a 10 Hz bipolar pulse trains (Table 2). The onset of morphologic response is dose–dependent. With increasing field strength at 60 Hz, the onset of morphologic change decreased from 6 days at 0.8 gauss to 1 day at 10 gauss (Table 1). Below 0.8 gauss, no morphologic response was observed after 12 days. When this ELF responsive cell line was plated in circular tissue culture dishes and exposed to ELF, there is a gradient of morphologic changes from center to the periphery of the dish. There is no change at the center of the dish and maximal change at the periphery (Fig. 3) correlating with gradient of induced electric field. When the exposure system is changed so there is uniform magnetic field as well as induced electric field, the pattern of morphologic change becomes randomly distributed throughout the flask (Fig. 4).

DISCUSSION

The geomagnetic field has been shown to be necessary for the fundamental biological activity of axonal ensheathment and myelination by the Schwann cell (1). Bioelectric perturbation of tissue has been shown to alter phenotypic expression of chondroblasts by promotion of alkaline phosphatase activity and alteration of the structure of proteoglycans in specific cartilaginous tissues (2). With that in mind, we assessed whether ELF magnetic field could induce morphologic change in a Schwann cell line that can be reliably observed. The morphologic change observed was dramatic and reproducible. The cell line was given the name ELF Responder 1 (ELFR1). The response was observed with several wave forms and the onset

of morphologic change varied systematically with ELF field strength. Observation of the morphologic changes induced by ELF exposure suggest that the field effect is likely to be mediated through the induced electric field with an apparent threshold between 0.75 and 0.8 gauss at 60 Hz.

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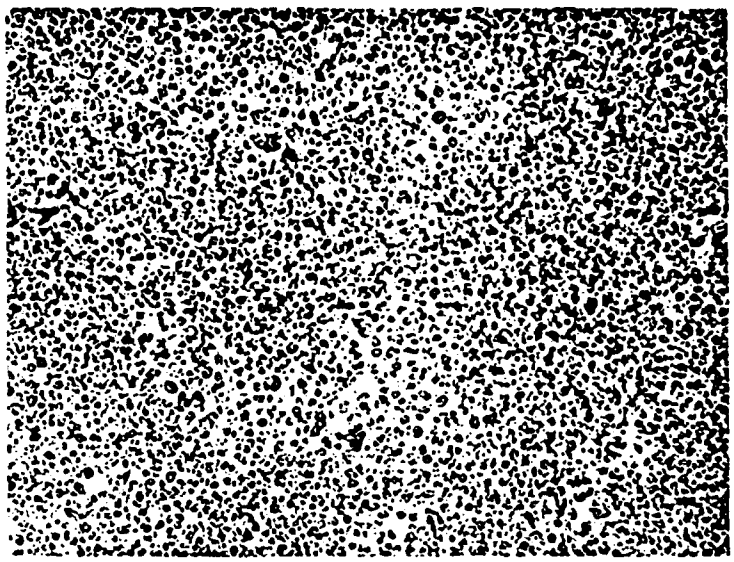

Fig. 1. ELFR1 exhibit round morphology without ELF exposure.

Fig. 2. ELFR1 exhibit bipolar morphology post ELF exposure.

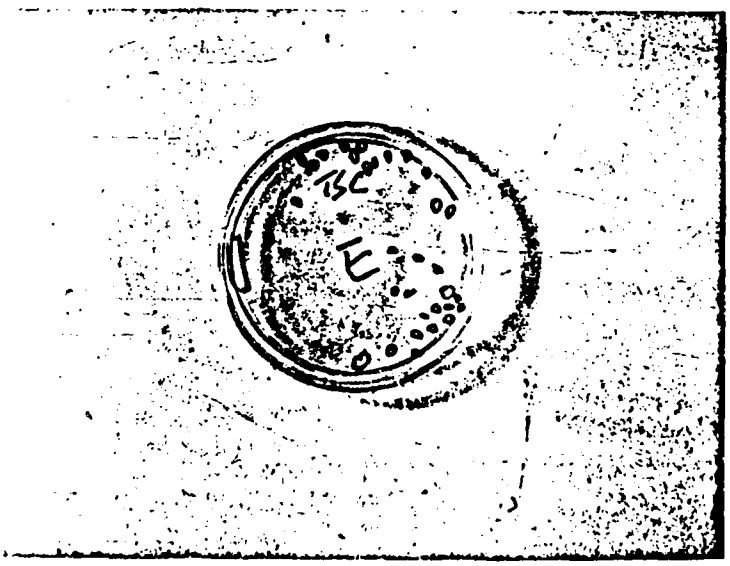

Fig. 3. Morphologic changes exhibited by ELFR1 increases from center to periphery of culture dish, porportional to magnitude of induced

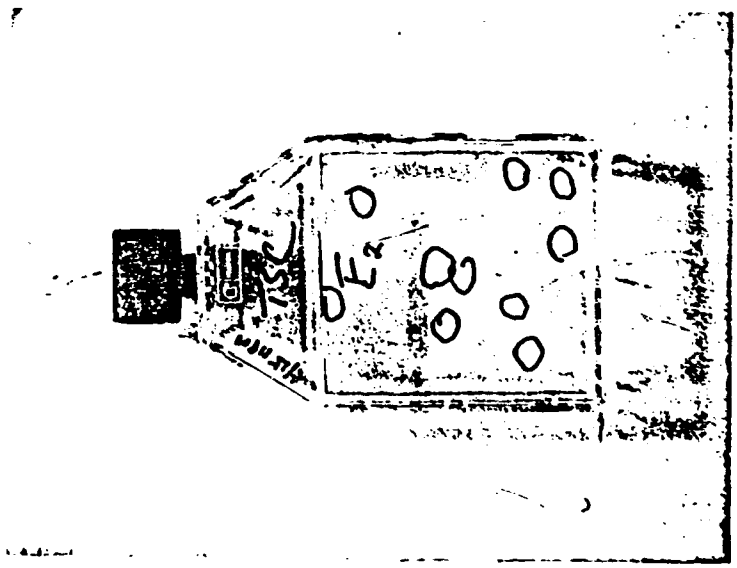

Fig. 4. Morphologic changes exhibited by ELFR1 is randomly distributed in uniform magnetic and induced electric field exposure configuration.

INTRACELLULAR CALCIUM SIGNALLING IN JURKAT E6-1 EXPOSED TO AN INDUCED 1 mV/cm, 60 Hz SINUSOIDAL ELECTRIC FIELD.

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ABSTRACT

Intracellular calcium signalling was monitored in Jurkat E6-1 cells following or during exposure to a 1 mV/cm, 60 Hz electric field induced by a 238 Gauss 60 Hz magnetic field from a large solenoid. Cells were exposed in protein-containing culture medium in the outer annular ring of a Falcon 3037 organ tissue culture dish. Fluo-3-loaded cells were stimulated with anti-CD3 monoclonal antibody at desired time points, and increases in fluorescence were monitored with a flow-cytometer. Cells were exposed at room temperature and at 37°C. In all cases, neither the calcium signal nor concomitant cell function from field-exposed cells differed significantly from controls.

INTRODUCTION

Several laboratories have reported alterations in the association of $^{45}Ca^{2+}$ with cultured tumor cells, normal lymphocytes, tissue preparations, or *in vivo* intact cat brains during or following exposure to different extremely low frequency (ELF) electromagnetic fields (for review see Lyle, 1991). This has suggested the possibility that ELF fields might alter transmembrane calcium signalling. Calcium signalling is a key pathway involved in the activation, differentiation, and proliferation of many cell types (Berridge and Irvine, 1989). Furthermore, it is associated with aspects of tumor promotion (Whitfield, 1990). Changes in $^{45}Ca^{2+}$ association, however, could be due to differences in binding at the cell surface and/or movement into the cell's interior. Jurkat is a human helper T-lymphocyte cell line that has been extensively used for studying calcium signalling pathways

(Weiss et al., 1984). We monitored an intracellular calcium signal stimulated by a monoclonal antibody against CD3, in Jurkat E6-1 following and during ELF field exposure. The cells were exposed to an induced 1 mV/cm, 60 Hz electric field in a solenoidal exposure regime that has been reported to alter $^{45}Ca^{2+}$ association with mitogen-stimulated rat thymocytes (Walleczek and Liburdy, 1990).

MATERIALS AND METHODS

The Jurkat clone E6-1 was grown in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin ("complete medium"). For experiments conducted in air, 10 mM HEPES was added to the complete medium to control pH. For monitoring of intracellular calcium, cells were loaded with 1 µM fluo-3 (Molecular Probes) via a 30-min incubation in the dark with the acetoxy-methyl ester. Cells were then washed three times in HEPES-buffered complete medium, and resuspended at 1-2 x 10^6/ml in HEPES-buffered complete medium, and placed 4 ml/dish into two annular-ring petri dishes (Falcon 3037 organ tissue culture dishes). The dishes were placed onto supports sitting in water priorly stabilized at the desired temperature, in a central reservoir within a water-jacketed closed container. Water at the appropriate temperature was constantly pumped from a circulating water bath through the walls of the incubation chambers. Temperatures were routinely checked before and after all field and control exposures.

One water-jacketed incubator was situated within the bore of a water-cooled solenoid, and the other at a distance of 9 feet from the solenoid. For a

solenoid vertical field of 60 Hz, 238 Gauss, the vertical 60 Hz value at the control site was 13.0 mG. Temperature variation between exposed and control dishes was 0.1°C or less. The vertical DC component, parallel to the AC, was 380 mG in the solenoid, and 480 mG at the control site. The solenoid (designed and built by Dr. Robert Liburdy at Lawrence Berkeley Laboratory; N = 400, 2.3 ohms, 20.1 mH) was energized by a 60 Hz power frequency current. Heat generated by the solenoid was effectively removed by its own water-jacketed, cooling housing (6 L/min). The 238_{rms} Gauss 60 Hz field used in these experiments induced an average 1 mV/cm, 60 Hz electric field in the medium of the annular ring of the organ tissue culture dishes. The associated current density was 16 $\mu A/cm^2$, with conductivity of the medium measured at 1.7 S/m.

Petri dishes were allowed to equilibrate in place on their respective water bath holders for at least 30-min, with the temperature set to 24 or 37 C. Then, using a 1-min stagger, fluo-3-loaded cells were placed into medium at the desired temperature and immediately poured into control and exposure sites. For 30-min pre-exposure experiments, maintaining the 1-min stagger, the petri dishes were removed, cells were quickly resuspended, placed to test tubes, and stimulated with a murine monoclonal antibody against human CD3. For simultaneous exposure during calcium signalling, cells were stimulated with anti-CD3 at the same time they were placed into the field and control sites, and 0.3 ml samples were taken directly from the petri dishes for analysis at time points up to one hour. Samples were analyzed on a Becton Dickenson FACScan flow-cytometer for the number of cells out of 10,000 examined showing fluorescence intensity above that of unstimulated fluo-3 loaded cells (Sei and Arora, 1991).

Significance of changes between field and control samples was determined within a single experiment using Kolmogorov–Smirnov statistics, or between groups of experiments via 2 tailed Student's t-tests or ANOVA.

RESULTS AND DISCUSSION

Figure 1 shows the anti-CD3 stimulated calcium signal time course over a 10-min period after cells were exposed to an induced 1 mV/cm, 60 Hz electric field for thirty minutes. Results are the means of four experiments normalized to the two minute peak of the medium value. As compared to the control situation, no significant changes within individual experiments or between the groups of experiments occurred. Prior experiments using a $^{45}Ca^{2+}$ endpoint noted a rapid effect by a field close to calcium ion resonance conditions, that was present during exposure but not detected following a 30-min pre-exposure (Lyle et al., 1991). Consequently, we then conducted the same experiment by taking samples during field exposure. In addition, two phases of calcium signalling are being examined in our system. There is a rapid release of intracellular calcium stores, which accounts for most of the initial peak, then a longer plateau phase which is maintained by movement of calcium across the plasma membrane (data not shown). It is conceivable that a field effect might not occur during the initial peak, but then be noted during the plateau phase. Thus we extended the assay period during the field exposure out to one hour. As seen in Figure 2, for a normalized group of four experiments, no significant changes occurred over a 1-h time course during constant field exposure. Another possible contributor to a

lack of an effect could be that the assays were being performed at room temperature. We then conducted the same experiment at 37°C, as seen in Figure 3. For this experiment, no significant changes were noted between field and control. A rapid loss of the dye was observed due to the 37°C incubation, however, which has been attributed in some cells to an anion transport system which actively excretes the dye (Di Virgilio et al., 1990). This, then, was a way to concomitantly look at cellular function during the 1-h period of calcium signalling monitoring. Figure 4 shows the percent of cells over time which lost fluorescence below the fluo-3 loaded cell baseline. Again, no significant changes were observed between field and control samples.

Since individual cell sensitivity to electric field stimulation has been reported (Binderman et al., 1985), it may be that the rapidly-proliferating, fully-transformed cell line Jurkat is insensitive to modulation by the field used in this study, whereas partially-activated normal cells such as thymocytes are sensitive (Walleczek and Liburdy, 1990). Also, calcium modulation at the surface of the cell might be affected in Jurkat whereas intracellular signalling processes might not be. Experiments are proceeding with Jurkat and also mitogen activated thymocytes in order to address these additional questions.

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Figure 1. Time course of calcium signal in Jurkat E6-1 cells after a 30-min exposure to a 238 Gauss sinusoidal 60 Hz field inducing a 1 mV/cm electric field in the culture medium. Results are the normalized means of 4 exp ± 1 S.D. (n = 10,000 cells/exp).

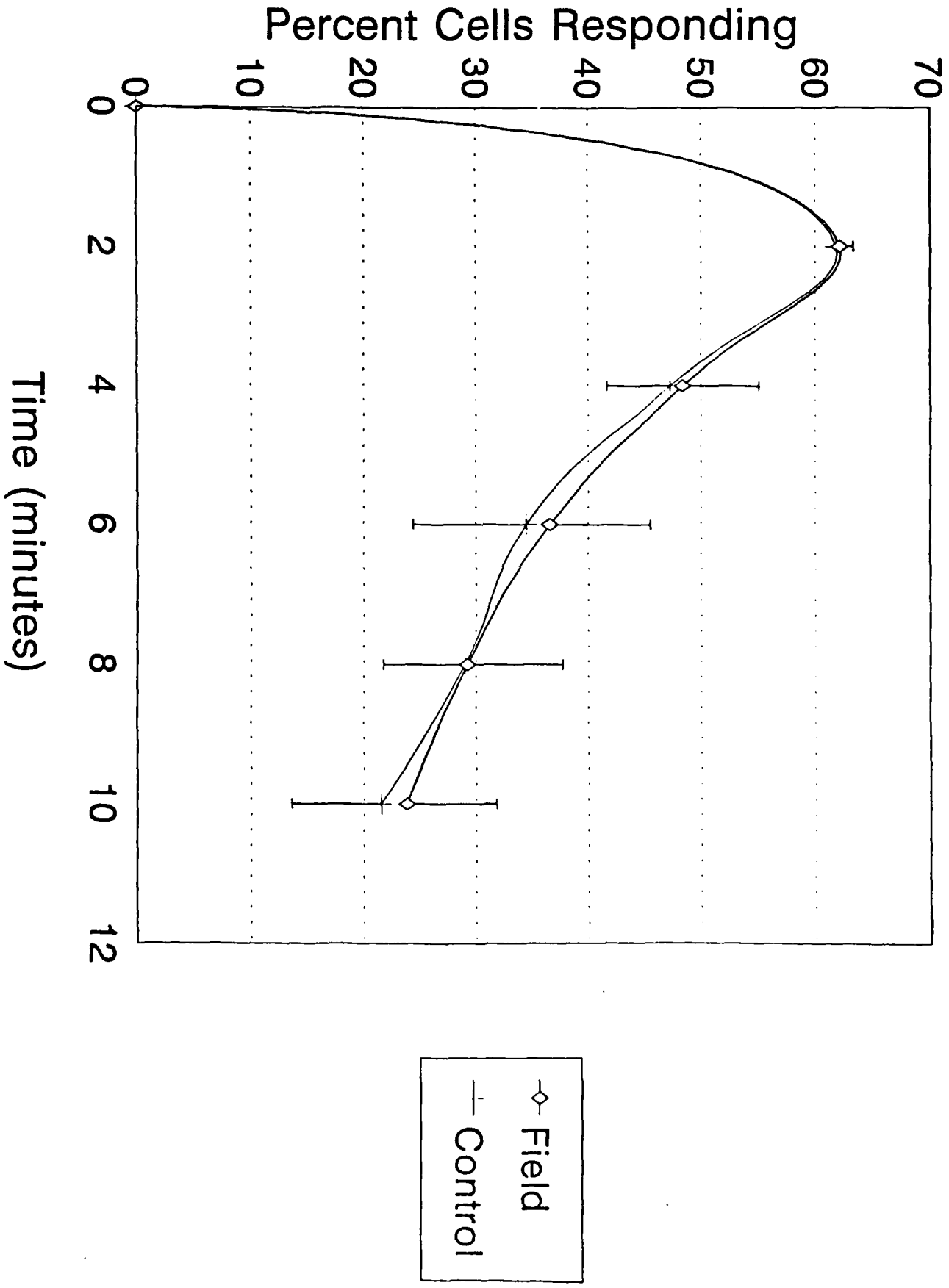
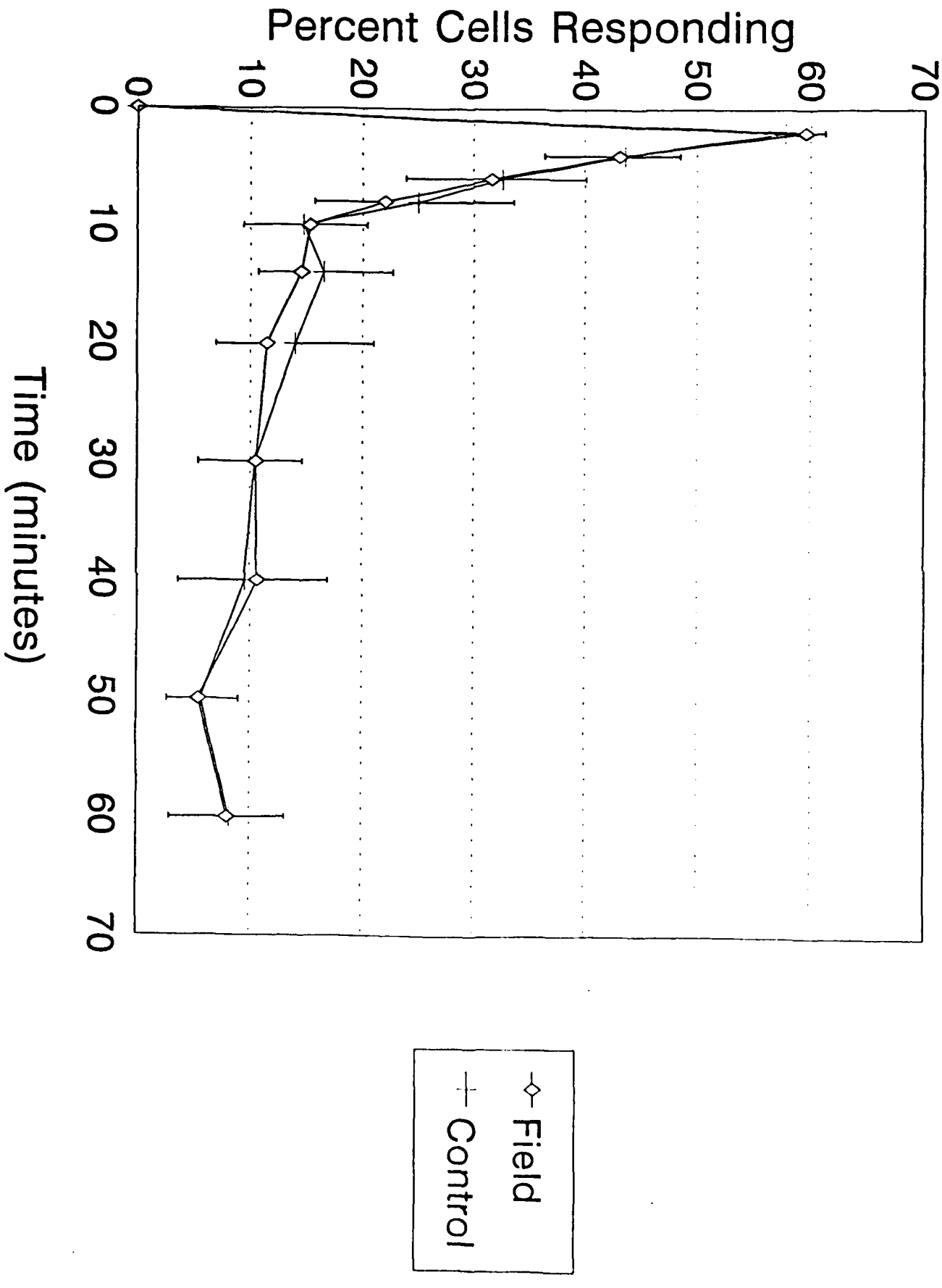

Percent Cells Responding
0
10
20
30
40
50
60
70
Time (minutes)
0
2
4
6
8
10
12
Field
Control

Figure 2. Time course of calcium signal in Jurkat E6-1 cells during exposure to a 238 Gauss sinusoidal 60 Hz field inducing a 1 mV/cm electric field in the culture medium. Results are the normalized means of 4 exp ± 1 S.D. (n = 10,000 cells/exp).


Percent Cells Responding
0
10
20
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40
50
60
70
Time (minutes)
0
10
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30
40
50
60
70
Field
Control

Figure 3. Time course of calcium signal in Jurkat E6-1 during exposure to a 238 Gauss sinusoidal 60 Hz field inducing a 1 mV/cm electric field in the culture medium, conducted at 37°C and 24°C. Each point is derived from the analysis of 10,000 cells.

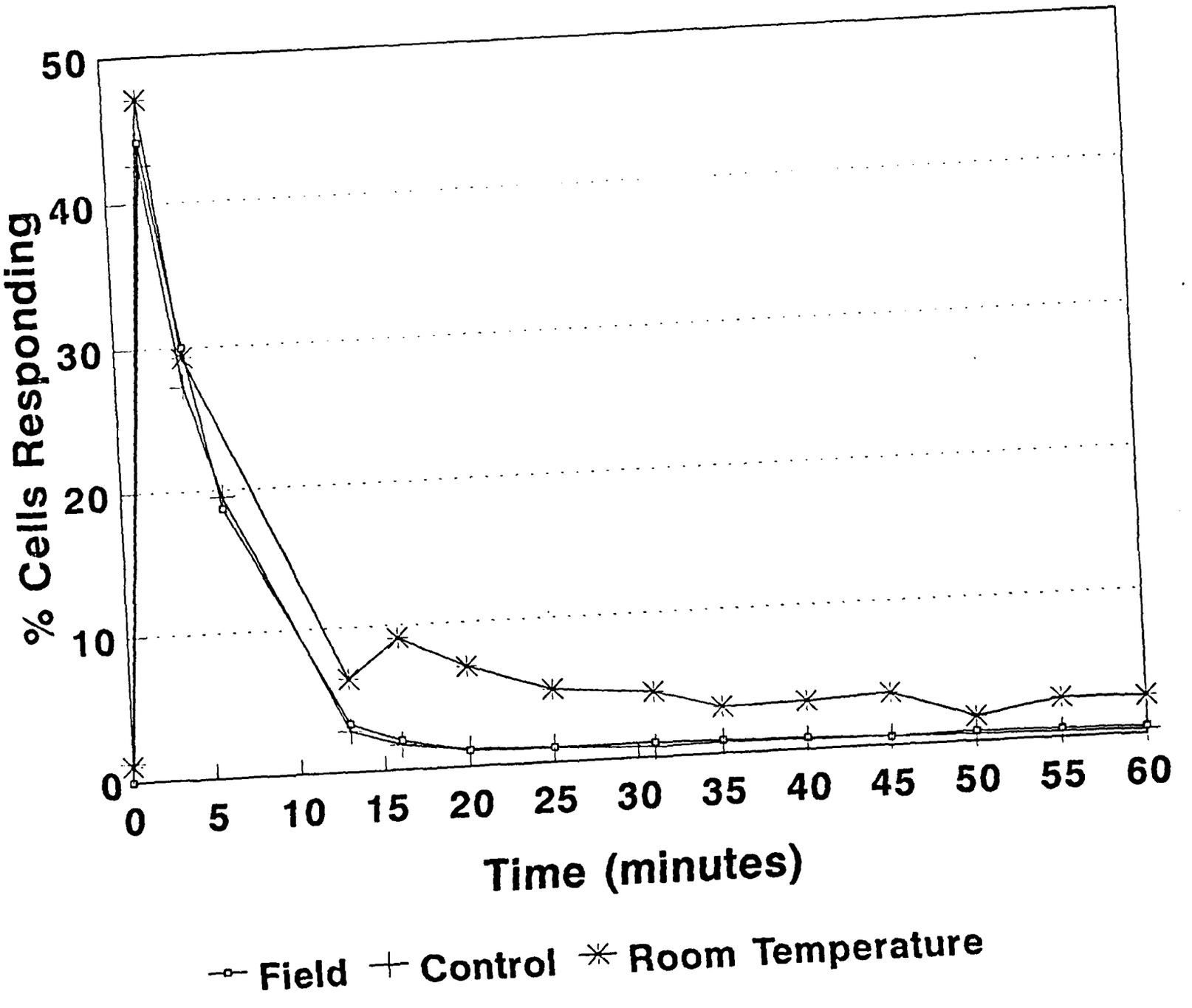
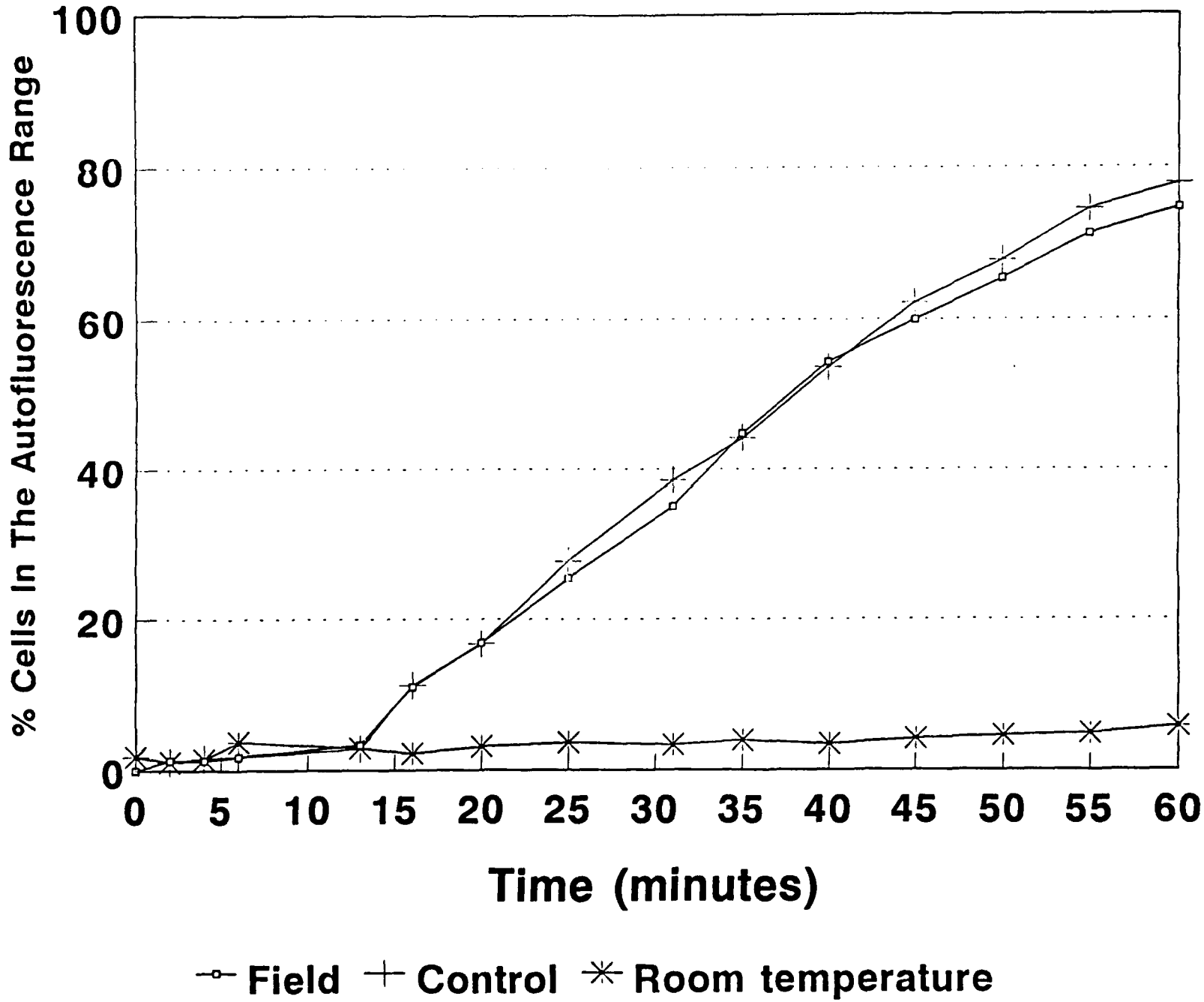

50
40
30
20
10
0
% Cells Responding
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (minutes)
Field
Control
Room Temperature

Figure 4. Time course of lose of baseline fluorescence in fluo-3-loaded Jurkat E6-1 cells exposed to a 238-Gauss sinusoidal 60 Hz field inducing a 1 mV/cm electric field in the culture medium, conducted at 37°C.


100
80
60
40
20
0
% Cells In The Autofluorescence Range
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (minutes)
Field
Control
Room temperature

PULSED ELECTROMAGNETIC FIELD EXPOSURE INTERFERES WITH ANTISENSE INHIBITION BOTH IN THE CYTOPLASMIC AND THE NUCLEAR COMPARTMENTS OF THE CELL

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ABSTRACT

Mitotically quiescent neonatal rat Schwann cells were transfected with a plasmid containing the SV-40 T antigen gene expressed under the control of the mouse metallothionein-I (MT-I) promoter. Antisense oligodeoxynucleotides directed to the transcription initiation site as well as the metal regulatory binding site of MT-I promoter did not exhibit sequence specific inhibition of cellular proliferation when exposed to pulsed electromagnetic field (PEMF). However, significant inhibition was observed in the absence of PEMF as demonstrated by [^{3}H] thymidine incorporation into DNA. Immunofluorescence staining with monoclonal antibody to SV-40 T antigen in the presence of PEMF also did not exhibit significant decrease in SV-40 T antigen protein suggesting that PEMF can interact with the cell at the levels of DNA and RNA and affect specific gene expression both in the nuclear and cytoplasmic compartments of the cell.

INTRODUCTION

Many biological responses have been reported in cells exposed to inductively coupled PEMF (1). PEMF has been used in the clinical setting for the treatment OF non-unions and pseudoarthroses of bone (2) via stimulation of bone growth and collagen synthesis in osteoblast-like cells (3). Specific waveforms between 15 and 4000 Hz have been shown experimentally to stimulate DNA synthesis (4).

PEMF was applied continuously to the transfected Schwann cells treated with oligodeoxynucleotide to ascertain any possible effects on base pairing by complementary oligodeoxynucleotides and their cellular nucleic acid targets. Interference with complementary base pairing was assessed by comparing the degree of antisense inhibition observed with and without PEMF exposure and the amount of SV-40 T antigen present in antisense oligodeoxynucleotides treated transfected Schwann cells immunocytologically stained with

monoclonal antibody to SV-40 T antigen.

MATERIALS AND METHODS

1. SYNTHESIS AND PURIFICATION OF UNMODIFIED OLIGODEOXYNUCLEOTIDES

Oligodeoxynucleotides were synthesized on the 1 uM scale using an automated DNA synthesizer (Applied Biosystem, Inc.) following the phosphoramidite protocol supplied by the manufacturer. After deblocking in concentrated ammonium hydroxide, the oligodeoxynucleotides were purified by thin layer chromatography using precoated silica gel 60 plates (EM Science). The full length oligodeoxynucleotides were extracted and resuspended in sterile water and concentration determined by spectrophotometry at 260 nm.

2. TARGET SITES OF OLIGODEOXYNUCLEOTIDES

Oligodeoxynucleotides were synthesized to the following target sites on the fusion plasmid pMtsv.neo containing the SV-40 T antigen gene under the control of the mouse MT-I promoter inserted into pMK-TK (HSV thymidine kinase).

Oligo 1: CAG TGG TGC TGA AGT TGC (Antisense to 5'terminus of SV-40 T antigen mRNA)

Oligo 2: TGG GAA ACG CGG GCC TGA (Antisense to metal regulatory site of MT-I promoter on plasmid DNA)

Oligo 3: TAC CGA AGC ATG GGG CCG (Antisense to 5'terminus of TK mRNA)

Oligo 4: GTC ACC ACG ACT TCA ACG (Sense to 5'terminus of SV-40 T antigen mRNA)

Oligo 5: 18 mer random mixed oligodeoxynucleotide

3. TRANSFECTED SCHWANN CELL CULTURE AND ANTISENSE INHIBITION PROTOCOL

Stably transfected Schwann cell line were grown routinely in DMEM containing 10% heat-inactivated FBS. Approximately 2000 cells were initially seeded into each of the 96 well

microtiter plates. On day 3 and day 5, the cultures were changed to fresh medium supplemented with the appropriate oligodeoxynucleotides.

4. CELL HARVESTER ASSAY OF [^{3}H] THYMIDINE UPTAKE

For thymidine uptake assay, [^{3}H] thymidine was added into culture on day 8. After 24 hours, the cells were washed, collected on filters and [^{3}H] thymidine incorporation determined by liquid scintillation counting.

5. INDIRECT IMMUNOFLUORESCENCE

SV-40 T antigen was detected by indirect immunofluorescence (5) on day 9 of culture, by employing monoclonal antibody to SV-40 T antigen (6) as the primary antibody. The fluorescein conjugated goat anti-mouse IgG antibody was used as the secondary antibody.

6. PEMF EXPOSURE

Transfected Schwann cells were exposed continuously to PEMF using a Biosteogen Model CU-204 (Electro-Biology, Inc.) 15 Hz pulse train signal generator two hours post treatment with oligodeoxynucleotides.

7. AUTORADIOGRAPHY

Oligodeoxynucleotides were radio-labeled by terminal transfer using Dupont/NEN 3'end labeling system and [^{35}S] dATP. On day 3, Schwann cell cultures initially seeded on cover slips were incubated with [^{35}S] labeled oligodeoxynucleotides for 2 hours. The cells were washed, fixed in 4% paraformaldehyde. The cover slips were then coated with Nuclear Track Emulsion and exposed at room temperature for 2 days. After exposure, the coated cultures on coverslips were developed and stained with hematoxylin.

RESULTS

Table 1 shows the effect of 2.8 uM oligodeoxynucleotides on [^{3}H] thymidine uptake of

transfected Schwann cells with or without PEMF exposure. Antisense oligodeoxynucleotides exhibited 12% to 32% inhibition of thymidine uptake while sense and random mixed control oligodeoxynucleotides exhibited 0% to 11% inhibition without PEMF exposure. When the cultures were exposed to PEMF, the antisense inhibition was antagonized, ie., there was no difference in [^{3}H] thymidine uptake between antisense oligodeoxynucleotides treated cultures and control oligodeoxynucleotides treated cultures.

Indirect immunofluorescence staining for SV-40 T antigen visually demonstrated the abolition of antisense inhibition by PEMF exposure. The nuclear fluorescence corresponding to the SV-40 T antigen was decreased by antisense oligodeoxynucleotides targeted to both the nuclear (Fig. 1b) and cytoplasmic (Fig. 2b) compartments of the transfected Schwann cells. But, with PEMF exposure (Fig. 1a & 2a), there was no evidence of decreased nuclear fluorescence. The immunofluorescence data correlated well with effect of PEMF exposure on [^{3}H] thymidine uptake.

Localization of nuclear and cytoplasmic targeted antisense oligodeoxynucleotides were performed by autoradiography of the respective antisense oligodeoxynucleotides. The antisense oligodeoxynucleotide targeted to the nucleus of the cell (Fig. 3a) gave a nuclear pattern of cell labeling whereas the antisense oligodeoxynucleotide targeted to the cytoplasmic compartment of the cell (Fig. 3b) gave a perinuclear pattern of cell labeling. This demonstrated that both nuclear and cytoplasmic targeting antisense oligodeoxynucleotides were able to complement with their appropriate targets both in the nuclear and cytoplasmic compartments of the cell.

DISCUSSIONS

Sequence specific inhibition of SV-40 T antigen expression by antisense oligodeoxynucleotides targeted to different sites of the fusion plasmid pMt sv.neo were decreased

by exposure to PEMF. This was demonstrated by utilizing two different assays, [3H] thymidine uptake and indirect immunofluorescence. This suggested that PEMF exposure can interfere with antisense inhibition both in the nuclear and cytoplasmic compartments of the transfected Schwann cells. Thus, it is possible to conjecture that PEMF can interact and potentially modify nucleic acids binding and their interactions with other macromolecules both in the nuclear and cytoplasmic compartments of the cell.

As a further demonstration that PEMF exposure can modify antisense inhibition to both nuclear and cytoplasmic compartments of the cell and that the antisense oligodeoxynucleotides can indeed complement with their nuclear and cytoplasmic nucleic acid targets, we radio-labeled oligodeoxynucleotides that were targeted to both the nuclear and cytoplasmic compartments of the cell and performed autoradiography. The labeled oligodeoxynucleotides that were targeted to the nuclear compartment of the cell exhibited localization of label primarily to the nucleus of the transfected Schwann cells after two hours incubation. The labeled oligodeoxynucleotides that was targeted to the cytoplasmic compartment of the cell exhibited perinuclear pattern of labelling after two hours of incubation. The autoradiographic results confirmed that antisense oligodeoxynucleotides can be targeted to both the nuclear and cytoplasmic compartments of the cell. Therefore, it is possible to conclude that in the thymidine uptake and immunofluorescence studies, PEMF exposure interferes with antisense inhibition both in the cytoplasmic and nuclear compartments of the transfected Schwann cell.

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FIGURE LEGEND

Table 1. Effect of 2.8 uM oligo deoxynucleotides on 3H thymidine uptake of transfected Schwann cells with and without PEMF exposure.

Figure 1. Indirect immunofluorescence microscopy of transfected Schwann cells treated with Oligo 1, antisense to 5'terminus of SV–40 T antigen mRNA. a) with PEMF exposure b) without PEMF exposure.

Figure 2. Indirect immunofluorescence microscopy of transfected Schwann cells treated with Oligo 2, antisense to metal regulatory site of MT–I promoter on plasmid DNA. a) with PEMF exposure.

Figure 3. Autoradiography of transfected Schwann cells with 35S labeled a) Oligo 2

b) Oligo 1.

TABLE 1

EFFECTS OF 24 nM OLIGODEOXYNUCLEOTIDE ON ^{3}H THYMIDINE UPTAKE OF TRANSFECTED SCHWANN CELLS WITH OR WITHOUT PEMF EXPOSURE

	n	MEAN(X10^3)	SD(X10^3)	%Inhibit.
0 Oligo	12	148	16	0
Oligo 1	12	100	14	32
Oligo 2	12	127	17	14
Oligo 3	12	131	16	12
Oligo 4	12	132	15	11
Oligo 5	12	132	22	11
PEMF				
0 Oligo	12	149		0
Oligo 1	12	141		5
Oligo 2	12	131		12
Oligo 3	12	127		15
Oligo 4	12	127		15
Oligo 5	12	127		15

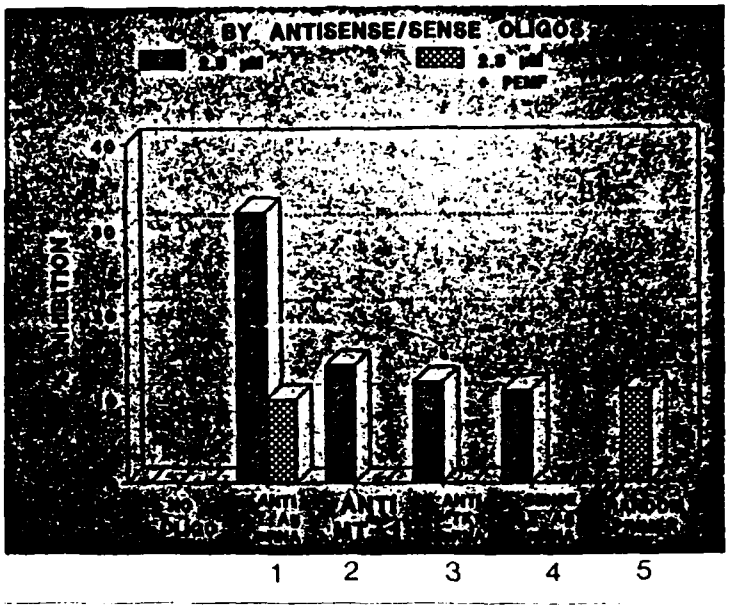


FIG. 1 a b

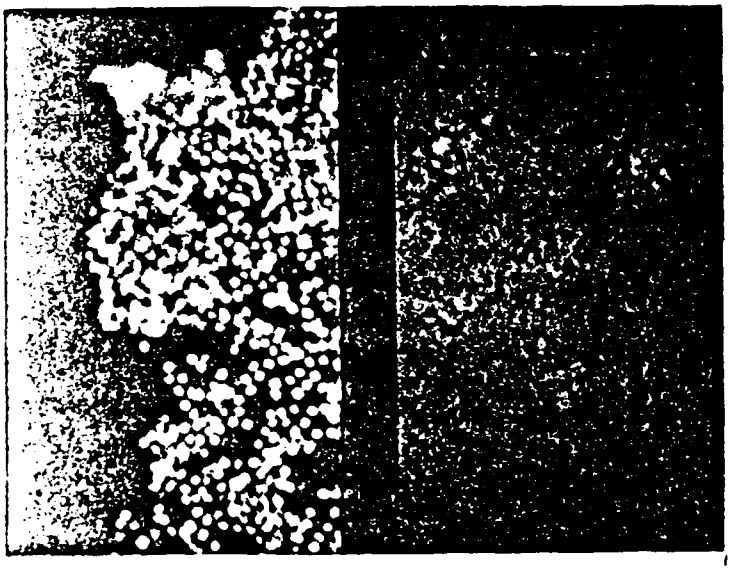


FIG. 2 a b

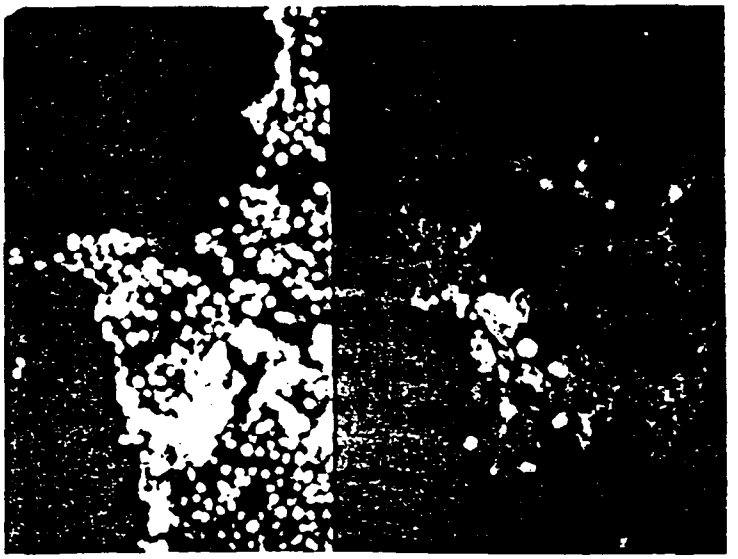


FIG. 3a

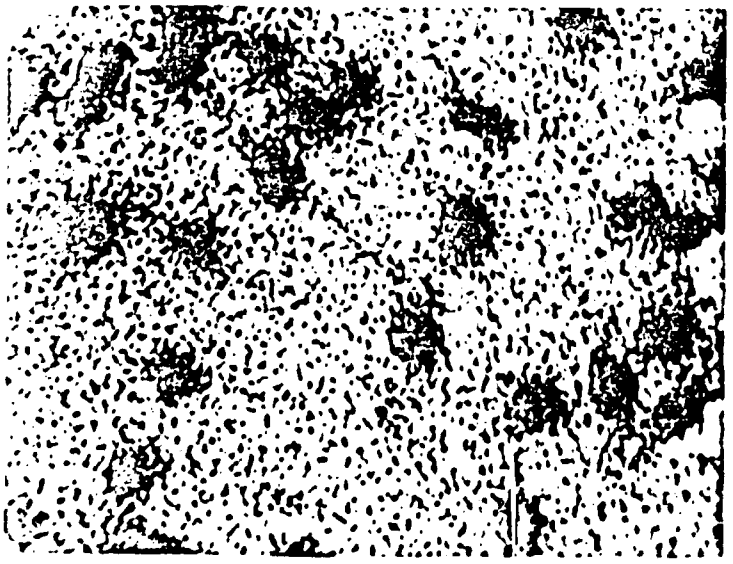
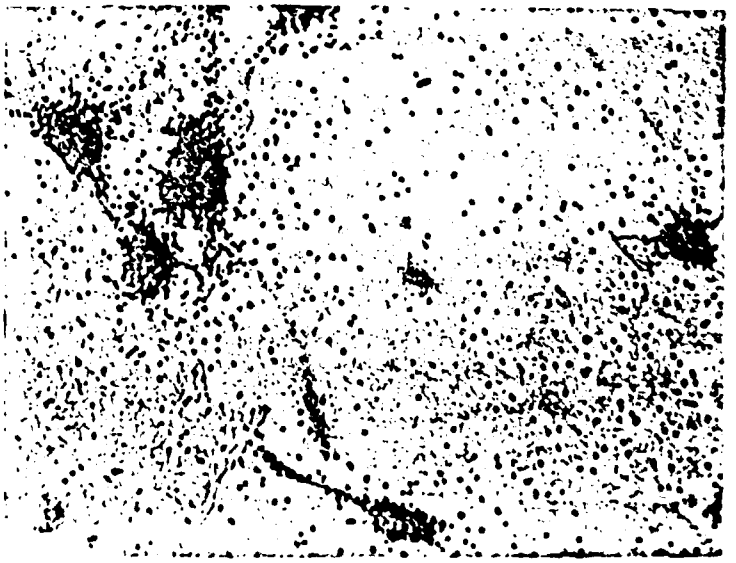


FIG. 3b



THE EFFECTS OF A SINGLE INTRAOPERATIVE IMMERSION IN VARIOUS CHEMICAL AGENTS AND ELECTROMAGNETIC FIELD EXPOSURE ON ONLAY BONE GRAFTS TO THE FACIAL SKELETON

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ABSTRACT

Bone grafts have been used extensively in craniomaxillofacial surgery. When bone is used as an onlay graft to alter facial contour, the volume of graft maintained is unpredictable. Therefore, any intervention making graft survival more predictable and permanent would find wide application in plastic and reconstructive surgery. We have, therefore, examined the effect of extremely low frequency (ELF) electromagnetic field exposure on onlay bone grafts to the facial skeleton in the rabbit model. Concurrently, we also assessed the effectiveness of a single application of chemical agents to bone grafts intraoperatively. These stable chemical agents were chosen because they induce *in vitro* the same second messengers which mediate the actions of various growth factors that are involved in osteogenesis in osteoblast cultures. Twelve adult male New Zealand white rabbits were used in this pilot study with two rabbits in each treatment group. Each rabbit underwent harvest of two iliac crest (endochondral bone) and two calvarial (membranous bone) grafts of known volume, measured directly by a caliper. The grafts were then implanted subperiosteally onto the rabbit snout after a single fifteen minutes immersion in Dubecco Modified Eagle's Medium (DMEM) with one of the following agents: 0.1 uM forskolin, 0.5 uM phorbol 12-myristate 13-acetate (PMA) 10 uM calcium ionophore A23187 or 0.4 uM beta-glycerol phosphate. The control grafts were immersed in DMEM alone for fifteen minutes. Grafts in ELF experiment were immersed in phosphate buffered saline for fifteen minutes. Post recovery, they were exposed for two to six hours per day, five days per week for twelve weeks to 60 Hz CW at one gauss. At the end of twelve weeks, the rabbits were sacrificed, the final graft volume measured by caliper and the percent of grafts remaining calculated. In all treatment groups, resorption was less for the membranous bone grafts than for the endochondral bone grafts. All treatment groups had a greater percent of graft volume remaining at the end of twelve

weeks than control grafts. The treated membranous grafts all exhibited final volumes greater than initial graft volumes with beta-glycerol phosphate treatment having the largest effect on final graft volume. ELF exposed grafts appeared to exhibit a dose-response relationship.

INTRODUCTION

Bone is one of the few tissues in the human body that possess the capability of regeneration post injury. Naturally occurring proteins which participate in the healing process have been known to exist from the time epidermal growth factor was isolated. Transforming growth factors, platelet-derived growth factor and fibroblast growth factors have all been shown to affect bone metabolism in cell culture[1].

Bone grafts have been used extensively in craniomaxillofacial surgery. When onlay bone grafts are used to alter facial contour, the volume of graft maintained is unpredictable. Any intervention making graft survival more predictable and permanent would have wide clinical applications. Electrical stimulation of onlay bone grafts have been reported to decrease graft resorption[2], but it inherently has several disadvantages when compared with electromagnetic field exposure. All electrodes are subject to some electrolysis which have deleterious effect on osteogenesis.[3] In addition, the electrode-stimulated bone formation is spatially limited due to the small size of the electrode compared to the repair site and would necessitate open reduction with its attendant surgical hazards and complications.

Specific changes have been shown following exposure of cells to electromagnetic fields in the extremely low frequency (ELF) range. Perturbations from normal biological processed have been observed in changes of ion flux, mRNA synthesis and DNA synthesis[4-5]. ELF fields have also been used clinically for the treatment of non-union fractures and arthodeses[6].

Therefore, the effect of ELF electromagnetic field exposure on onlay bone grafts to the facial

skeleton was examined in the rabbit model. Concurrently, we also assessed the effectiveness of a single intraoperative immersion of onlay bone grafts to stable chemical inducers of various second messengers known to be involved in osteogenesis in *in vitro* osteoblast culture systems.[7-8]

MATERIALS AND METHODS

Twelve adult male New Zealand white rabbits were used in this pilot study with two rabbits in each treatment group. Each rabbit underwent harvest of two iliac crest (endochondral bone) and two calvarial (membranous bone) grafts of known volume, measured directly by a caliper. The grafts then were implanted subperiosteally onto the rabbit snout after a single fifteen minute immersion in Dubecco Modified Eagle's Medium (DMEM) with one of the following agents: 0.1 uM forskolin, 0.5 uM phorbol 12-myristate 13-acetate, 10 uM calcium ionophore A23187 or 0.4 uM beta-glycerol phosphate. The control grafts were immersed in DMEM alone for fifteen minutes. Grafts in ELF experiment were immersed in phosphate buffered saline for fifteen minutes and were exposed to two to six hours per day, five days per week for twelve weeks of 60 Hz continuous waves at one gauss in plastic cages placed inside Helmholtz coils. All rabbits received food and water *ad lib.* and alternating twelve-hour light and dark cycle in temperature and humidity controlled animal facilities. At the end of twelve weeks, the rabbits were sacrificed and the final graft volume measured and the percent of graft remaining were calculated.

RESULTS

In all treatment groups, membranous bone graft's resorption was much less than endochondral bone graft resorption (Table 1). All treatment groups had a greater percentage of graft volume remaining at the end of twelve weeks than control grafts. The treated membranous grafts all exhibited final volumes greater than initial graft volumes with beta-glycerol phosphate

treatment having the largest effect on final graft volume. ELF exposed grafts appeared to exhibit a dose-response relationship of the final graft volume with the duration of ELF exposure (Figure 1).

DISCUSSION

The resorption of membranous bone grafts were less than endochondral bone grafts in our rabbit model which is in agreement with the literature.[9] It is interesting to note that membranous bone grafts in most treatment groups had appreciably larger final graft volume. This suggest that *de novo* bone deposition may have occurred.

The cellular and biochemical mechanisms of bone healing in general are just beginning to be understood [10-11]. The basic mechanism and initiating factors in cellular response to ELF fields are not well understood. Showing effects of chemical agents and ELF electromagnetic fields in this rabbit snout model may lead to an increased understanding of osteogenesis that may have wide applications in orthopedic surgery, plastic surgery and treatment of metabolic bone disorders.

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INTRACELLULAR CALCIUM SIGNALLING BY T-LYMPHOCYTES EXPOSED TO 60-Hz MAGNETIC FIELDS. Daniel B. Lyle, Lee A. Rosen, Thomas A. Fuchs, and Mays L. Swicord. CDRH, Food and Drug Administration, Rockville, MD 20857.

To explore possible biochemical mechanisms whereby weak electromagnetic fields might affect immune cells or developing cancer cells, we studied intracellular calcium signalling in Jurkat E6-1 human T-leukemia cells and in rat thymocytes during exposure to 60-Hz magnetic fields. Cells were labeled with the intracellular calcium-sensitive fluorescent dye fluo-3, stimulated with mitogens or a monoclonal antibody against the cell surface structure CD3 (associated with ligand-stimulated T-cell activation), and analyzed on a FACScan flow-cytometer for increases in intensity of emissions in the range of 515-545 nm. Cells were exposed during or before calcium signal-stimulation to a 60 Hz magnetic field superimposed upon a static magnetic field, "tuned" to $^{40}Ca^{2+}$ according to the theory proposed by V.V. Lednev (Bioelectromagnetics 12:71-75; 1991). Samples consisted of 5-ml volumes of cells in normal culture medium, placed in 50-ml centrifuge tubes in two vertically oriented solenoids in a single circulating flow-through water bath with water maintained at 24°C or 37°C. The total DC magnetic field of 782 mG was aligned 17.5° off the vertical axis. The 60 Hz AC field was adjusted to 1.5 Gauss along the solenoid axis, thus providing the appropriate component (1.44 Gauss) along the total vector of the DC field. When Jurkat was preexposed for twenty minutes to the field at 24°C, then stimulated by anti-CD3 and exposed to the field for twenty more minutes, no significant change occurred between field and control calcium signals. When calcium signal stimulation plus field exposure occurred simultaneously over a ten minute period at 37°C, again no field effect was observed. Similar data was obtained using normal rat thymocytes stimulated with optimal concentrations of mitogen. These experiments demonstrate that intracellular signalling in Jurkat E6-1 and rat thymocytes was not affected by a "Lednev Field" tuned to calcium, when culture and calcium signal-stimulation was optimal. Further experiments are proceeding to examine the effect of the field on calcium signalling in these cells under suboptimal conditions of 1) mitogen or antibody concentration, and 2) of external calcium availability.

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ELF exposure (FIGURE 1).

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72: 7N

Comparison of the Effect of ELF on Total RNA Content in Normal and Transformed Human Cells

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INTRODUCTION

Various reports in the literature have indicated that extremely low frequency (ELF) electromagnetic radiation exposure can have different effects on gene transcription in human cells *in vitro*.[1–4] Therefore, we investigated the effect of ELF on cellular transcription by flow cytometry on intact human cells. Flow cytometry enables us to estimate simultaneously with high accuracy the relative quantities of a variety of cellular constituents, including total RNA content.[5]

MATERIALS AND METHODS

HL-60, a human promyelocytic leukemia cell line, and Daudi, a human lymphoma cell line, were obtained from ATCC and were routinely cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum. Normal peripheral human lymphocytes were routinely cultured in chromosomic medium containing 2% per ml phytohemagglutinin (PHA). Equal numbers of cells in suspension were placed in 10 ml Corning T-75 tissue culture flasks containing fresh medium one hour prior to exposure. Cells in a 5% CO_2 incubator at 37° C were exposed for 30 and 60 min to 60 Hz continuous waves at 1 gauss or to pulsed electromagnetic fields (PEMF) produced by an EBI generator. After exposure, the cells were immediately fixed in 50% ethanol, incubated with acridine orange, and processed for flow cytometric analysis. For each analysis of data, 10,000 cells were counted and their RNA fluorescence was displayed as a histogram.

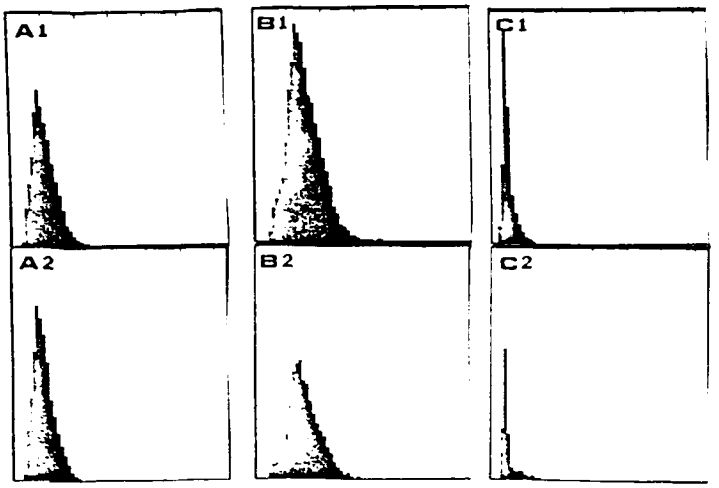


FIGURE 1. Effect of 60-Hz continuous wave exposure for 30 min on the total RNA fluorescence of Daudi, HL-60, and human peripheral lymphocytes. Increasing RNA fluorescence corresponds to a shift to the right on the abscissa—A1: HL-60 exposed, A2: HL-60 control; B1: Daudi exposed, B2: Daudi control; C1: lymph exposed, C2: lymph control.

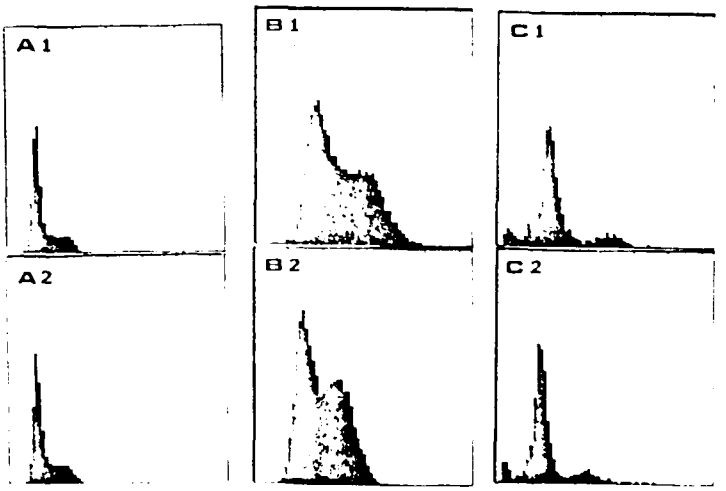
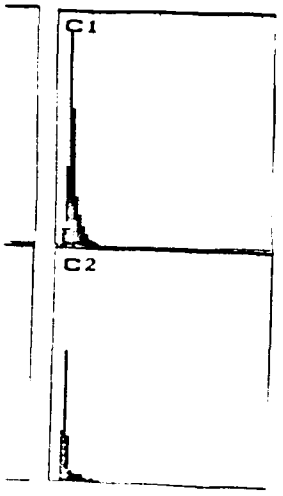


FIGURE 2. Effect of PEMF exposure for 30 min on the total RNA fluorescence of Daudi, HL-60, and peripheral human lymphocytes. Increasing RNA fluorescence corresponds to a shift to the right on the abscissa—A1: HL-60 exposed, A2: HL-60 control; B1: Daudi exposed, B2: Daudi control; C1: lymph exposed, C2: lymph control.



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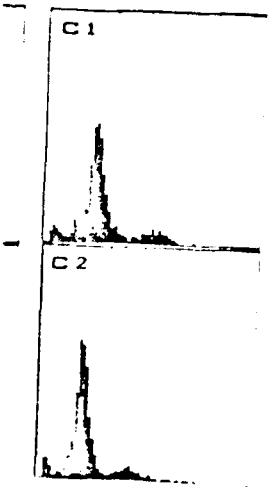
lymph control.



tal RNA fluorescence of Daudi.
NA fluorescence corresponds to a
HL-60 control; B1: Daudi exposed.

RESULTS

Total RNA fluorescence increased for PHA-stimulated normal peripheral human lymphocytes and Daudi lymphoma cells that were exposed to PEMF. However, no appreciable effects on total RNA fluorescence were seen in HL-60 promyelogenous leukemia cells exposed to PEMF. The magnitude of the RNA fluorescence increase was greater in cells exposed to PEMF than in those exposed to 60 Hz.

DISCUSSION

It is interesting to note that ELF exposure apparently has different effects on HL-60 and Daudi cells. These experiments have been repeated several times with similar results, suggesting that the observed difference is real. Further experiments are needed to examine more closely the different effects of ELF on Daudi and HL-60 cellular transcription.

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EFFECTS OF ELF ON C-MYC ONCOGENE EXPRESSION IN NORMAL AND TRANSFORMED HUMAN CELLS

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Abstract: Cell cultures of the human lymphoma cell line, Daudi, were exposed to 2.5, 5, 7.5,or 10 Gauss extremely low frequency magnetic fields. Transcription of the genetic marker, c-myc, was determined in cultures exposed for various time periods. Increases of c-myc transcripts were detected in some exposed cell cultures. The increase of the transcripts appeared to show dependence both on the applied field and exposure duration.

INTRODUCTION

Epidemiological, animal, and in vitro studies suggest linkage between exposure to 60 Hz sinusoidal electromagnetic fields (EMFs) and incidence of cancer (see review in [4]). The main concern is exposure to transmission power lines and to several electric devices. The existing data are still controversial, as they do not fully support the idea of extremely low frequency (ELF) EMFs being a cancer promotor. The data also do not exclude this possibility. In addition, no mechanistic basis has been established for ELF as a cancer promoting agent; this leads to further controversy.

Exposure to ELF EMFs is known to increase the level of several gene transcripts. Reba Goodman and coworkers [2,5] showed changes in gene transcription in Drosophila salivary gland cells and in HL-60, a human promyelogenous leukemia cell line (beta-actin, histone 2B and c-myc). Jerry Phillips [3] reported increased transcription of the genes encoding c-myc, c-fos, c-jun, and protein kinase C in human cell lines. The utilization of various exposure systems and the characteristics of applied fields, as well as differences among biological models, lead to problems in comparing results. However, the above papers report increased gene transcription following exposure to ELF EMFs.

We focused our research on transcription of the c-myc oncogene. We have examined transcription in two transformed human cell lines (Daudi lymphoma and HL-60) and in normal human peripheral lymphocytes. Transcription was examined by Northern blot hybridization analysis. Exposure for 30, 45, 60, or 180 min. to 60 Hz continous waves at 1 gauss resulted in increased expression of c-myc oncogene expression in Daudi cells. No appreciable changes were noticed in c-myc expression in HL-60 and peripheral human lymphocytes. The presence of a chromosomal translocation involving the c-myc locus in the Daudi cells [6] be an explanation for the increased c-myc transcription in this cell line.

We have expanded our experiments on Daudi cells by introducing different exposure parameters. In the present experiments, cells were exposed to 2.5, 5, 7.5, or 10 gauss, 60Hz sinusoidal EMFs. Positive results were obtained.

MATERIALS AND METHODS

Human cancer cell lines were obtained from the American Type Culture Collection, Rockville, MD 20852, and cultured in RPMI-1640 medium with 20% fetal bovine serum. Cells were grown in an CO_2 incubator, 5% CO_2 to a density of $5x10^5$/ml, and resuspended at the same cell density in fresh medium 1 hour prior to ELF exposure. Cell suspensions were placed in 30 ml aliquots in Corning T75 tissue culture flasks and exposed for 15, 30, or 60 min. to 2.5, 5, 7.5, or 10 gauss (G) 60 Hz sinusoidal EMF in the incubator, 5% CO_2, at 37° C. Each exposure flask was accompanied by an identical control flask that was placed in the μ metal box in the same incubator. After exposure, total RNA was immediately extracted with guanidinium isothiocyanate and the RNA pelleted by CsCl step gradient ultracentrifugation. The purified total RNA samples were then run in a denaturing formaldehyde agarose gels and transferred to polymer membranes (Northern blot separation technique). Phosphorus-32-labelled DNA c-myc and beta-actin probes were used to hybridize against the Northern blots. The commercially available c-myc probe (Oncor, Inc.) showed specificity with RNA in our laboratory. However, commercial beta-actin (Oncor, Inc.) was not adequately specific, so we used a probe prepared at the Medical College of Virginia, Richmond. The RNA samples from both exposed and control cells were probed at the same time. Autoradiography was carried out at -70° C for 2-3 days. A laser scanning densitometer was used to quantitate the intensity of hybridization of the two probes. The ratio of densitometer values, exposed : control, was determined. Data are presented as that ratio. Values were normalized to densitometer values of beta-actin.

RESULTS

Analysis of Northern blots showed increased expression of c-myc oncogene following 60 min. exposures to ELF. The increase is apparently dose dependent, with the exposed : control ratios varying from 1.62 at 2.5 G and 1.75 at 7.5 G, to 2.67 at 10 G exposures. Consistent patterns of reactions were not observed after exposures at other applied fields and for other exposure durations. The results are presented in Table 1.

Table 1. Laser densitometric quantitation of c-myc expression normalized to beta-actin to compensate for difference in sample loading. Ratio exposed : control.

Exposure Time (min)	Applied Fields, G		
	2.5	7.5	10
15	1.16	0.90	1.29
30	1.34	1.01	1.42
60	1.62	1.75	2.67

Long exposure durations, i.e., 3, 6, or 12 hours, did not show any differences between exposed and control samples. This was an expected result, inasmuch as the gene transcripts are short-lived.

DISCUSSION

Previously published data on the exposure of normal and transformed human cell lines [1] showed increased c-myc expression following 1 hour exposure to 60 Hz sinusoidal EMF in Daudi lymphoma cells known to carry a translocation in c-myc locus on the chromosome. No differences in c-myc expression were observed in the HL-60 cells and in human peripheral lymphocytes. The expression of c-myc appeared to depend on the dose and on duration of the applied field. Although Northern blot analysis provided relative data about specific gene products, quantification is difficult. Some sources of variation include variations in dosimetry readings for exposure of cultures, in autoradiography, and in densitometry. Other methods should be employed to measure specific levels of transcripts, in order to reduce variability and to provide a clear basis for interpretation.

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